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Since the completion of the seventh volume of this Journal Dr. H. R. Ing, F.R.S., has resigned from the position of Press Editor. This position has involved heavy work in preparing papers for publication. The Editorial Board on behalf of the British Pharmacological Society wish to record their appreciation of his great services, which have made a major contribution to the success of the journal.

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# ANALGESIC AND OTHER PROPERTIES OF 3 : 3-DITHIENYLALKENYLAMINES

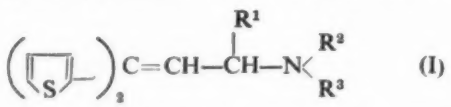
BY

A. F. GREEN

*From the Wellcome Research Laboratories, Langley Court, Beckenham, Kent*

(RECEIVED AUGUST 18, 1951)

Several 3 : 3-diphenylallylamines (Adamson, 1949) have shown moderate atropine-like, antihistamine, and local anaesthetic properties (White, Green, and Hudson, 1951). A number of allied series have also been investigated, among them dithienylalkenylamines (I) (Adamson, 1950).



(I)

These have been shown to have similar properties, but some are also powerful analgesics (Adamson and Green, 1950) and will be described in greater detail.

## METHODS

*Analgesia.*—The pain thresholds of groups of 10 or more 3- to 4-week-old rats were measured 30 minutes after subcutaneous injection of the analgesics, at each of two or more dose levels, by the heat and pressure methods described by Green and Young (1951). Activities relative to that of morphine, tested on the same occasion, were estimated from the regression lines relating log-dose and the probit of the proportion of animals in the group whose pain thresholds were at least twice that of a control group injected with saline.

In rabbits pain thresholds were measured with a simple analgesiometer constructed from a 20 ml. "Record" type syringe by soldering a brass wedge to the knob of the piston. The piston was lubricated with vaseline and set at the 20 ml. mark, after which the syringe was sealed with an occluded needle mount. Holding the barrel, pressure was applied to the forelimb of the rabbit through the brass wedge until an avoidance reaction was produced. When this occurred the volume of air in the syringe was recorded. The pressure in cm. of mercury was calculated from Boyle's law. The proportion of animals failing to respond to a pressure of 114 cm. of mercury, 60 minutes after subcutaneous injection of an analgesic, was used to calculate the ratios of activity, by probit analysis.

The results shown in Table II were obtained by injecting each compound at doses of 1, 3, and 9 mg./kg. into groups of 10 rabbits. The regression of the probit response on log-dose had a mean slope for all the drugs of 2.5, and the ED50 of morphine sulphate was approximately 2 mg./kg.

The syringe analgesiometer was also used on dogs. It was applied to the tip of each ear and to the skin of the foreleg over the radius. The proportion of dogs with a 100% increase in pain threshold was used as a basis of comparison. Durations of analgesia were determined by repetition of the test at half-hourly intervals up to two to three hours and hourly thereafter. The dogs were rarely used for more than one experiment; about 5% were rejected because their initial pain thresholds were too high.

*Breathing.*—Relative activity in causing a depression of respiratory movement was estimated in rats and rabbits from the relationship of dose to frequency of respiration. The latter was determined by recording the pressure changes in a tambour held against the belly of the animal. The minute volume was determined in unanaesthetized rabbits at rest by applying a face mask and recording the mean rate of air flow (Gaddum, 1941).

*Temperature changes* in the rabbit were measured by a thermocouple in the rectum connected to a recording galvanometer.

*Antihistamine action* was determined on guinea-pig ileum *in vitro* using the procedure described by White, Green, and Hudson (1951). Each of two concentrations of the compound and of mepyramine (the standard) was tested on spasm induced by histamine in each of four strips of ileum in random order, by adding them to the organ bath immediately after the effect of histamine had reached its peak. Relative activities were estimated from the mean percentage relaxation after three minutes.

*Atropine-like activity* was determined on isolated rabbit ileum, the procedure being the same as for antihistamine action but using carbachol $2.5 \times 10^{-7}$ to cause contraction of the muscle, and atropine sulphate as the standard of comparison. Mydriatic action was determined from the pupil diameters of groups of 10 mice, 30 minutes after intraperitoneal injection (Ing, Dawes, and Wajda, 1945).

## RESULTS

Fig. 1 shows the results of a preliminary exploration of the analgesic, atropine-like, and

antihistamine effects of the hydrochlorides of the dithienylalkenylamines (I). The tops of the columns indicate, on a logarithmic scale of ordinates, the activity found. The lower end of each column is the level below which no attempt was made to measure activity, and where no activity was observed a horizontal line is drawn in the chart. The black columns indicate the ratio of the analgesic activity of each compound to that of morphine sulphate in the rat by the pressure method ; the cross-hatched show the ratio of antagonism of carbachol on the rabbit intestine to that produced by atropine ; and the clear columns the ratio of antihistamine action on guinea-pig ileum to that of mepyramine. Fig. 1A shows the activities of a number of dithienylallylamines (I, $R^1=H$) with varying substituents on the amino group ($NR^2R^3$) ; Fig. 1B the effect of varying the substituent $R^1$ when $NR^2R^3$ is dimethylamino ; and Fig. 1C the effect of varying the amino substituent in dithienylbutenylamines (I, $R^1=Me$).

Variation of the substituent $R^1$ has a marked effect on analgesic activity, which is far greater where $R^1$ is methyl (butenylamine series) than where $R^1$ is hydrogen (allylamines) or any other substituent. Variation of $R^1$ has a smaller effect on atropine-like and antihistamine activities, which are approximately the same for the allylamine and butenylamine series. Fig. 1B indicates that, where $NR^2R^3$ is dimethylamino, antagonism to carbachol is greater when $R^1$ is hydrogen and antihistamine action when $R^1$ is ethyl.

Each of the activities is influenced by variation of the amino group $NR^2R^3$. The most active analgesic is the ethylmethylamino butenylamine, with an activity 1.7 times that of morphine sulphate.

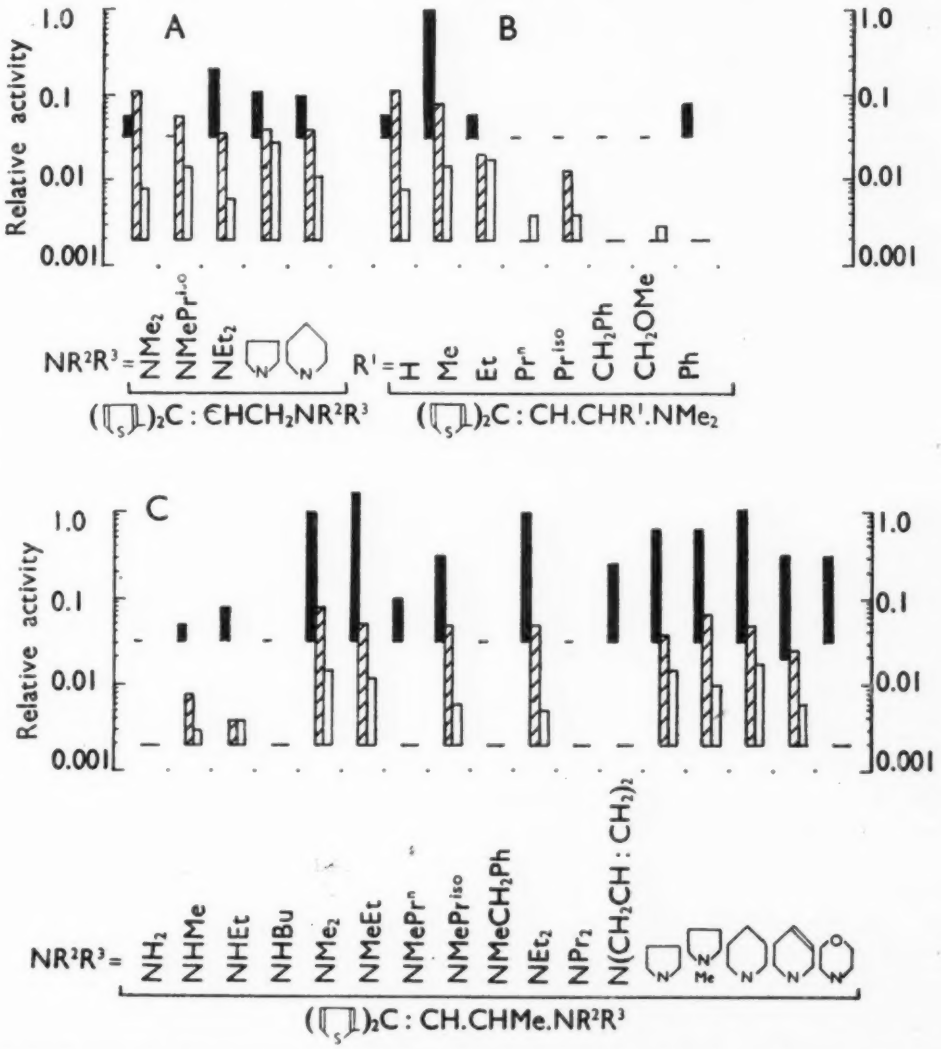



FIG. 1.—Relative activities of the dithienylalkenylamine hydrochlorides (I). The upper ends of the columns give the activity found. The lower ends are the levels below which no activities were measured, and when the activity of a compound did not exceed this a horizontal line is drawn. Black columns: analgesic activity in rats, morphine sulphate = 1·0. Cross-hatched columns: carbachol antagonism in isolated rabbit ileum, atropine sulphate = 1·0. Clear columns: antihistamine action in isolated guinea-pig ileum, mepyramine = 1·0.

Compounds with similar amino substituents (dimethylamino, diethylamino, pyrrolidino, and piperidino) are also powerful analgesics, but the activity decreases with reduction or increase in the dimensions of the amino substituents. In general, amino substituents favouring analgesic activity also favour carbachol and histamine antagonism, but to different degrees and with some notable exceptions (e.g. morpholino and diallylamino).

Those compounds with the greatest antagonistic action to carbachol caused in large doses marked mydriasis in mice, but they were only about 0.005 times as active as atropine. Local anaesthetic activity on the rabbit cornea equivalent to or greater than that of cocaine was shown by some substances ; they caused damage to the cornea and were not investigated further.

Five of the analgesics in the series were selected for further study. They are the butenylamine hydrochlorides (I, $R^1 = Me$) with $NR^2R^3$ as $NMe_2$, NMeEt, $NEt_2$, pyrrolidino, or piperidino, whose laboratory code numbers, used by others (Keele, 1952 ; Eddy and Leimbach, 1953), are respectively 338C48, 1C50, 191C49, 268C49, and 127C49. They will be referred to below by their amino substituents.

*Analgesia.*—The slopes of the regression lines relating the dose of the thienyl compounds to their analgesic effect (probit response) were not significantly different from that of morphine in any of the several tests using the pressure method which, when analysed, gave the relative potencies shown in Table I. By the heat method the activities of the ethylmethylamino and pyrrolidino compounds were, respectively, 1.0 (limits, p=0.95, 0.7–1.6) and 0.37 (0.26–0.55) times that of morphine sulphate. These values are less than those obtained by the pressure method (Table I). A similar difference in the potency ratios of pethidine: morphine by the two methods has been reported previously (Green and Young, 1951). The activities of the ethylmethylamino and pyrrolidino compounds when estimated from the probit of rats showing complete insensitivity to pressure stimuli were not significantly different from those obtained with smaller pressure stimuli.

TABLE I

ANALGESIC ACTIVITIES OF DITHIENYLBUTENYLAMINES (I, $R^1$=Me) IN THE RAT

The weighted mean values of several tests in which thresholds to pressure were determined 30 minutes after subcutaneous injection

| Amino Group | Number of Tests | Activity Ratio, Morphine Sulphate=1·0 | |
|---|---|---|---|
| | | Mean | Limits of Error, p=0·95 |
| Dimethylamino .. | 4 | 1·07 | 0·91–1·25 |
| Ethylmethylamino .. | 4 | 1·7 | 1·54–1·87 |
| Diethylamino .. | 2 | 1·0 | 0·83–1·10 |
| Pyrrolidino .. .. | 5 | 0·7 | 0·62–0·78 |
| Piperidino .. .. | 2 | 1·1 | 0·97–1·30 |

Estimates of the analgesic activities of three of the compounds *in rabbits* are shown in Table II. Although the limits of error are wider than those obtained in rats, the ratios are substantially the same.

TABLE II

ANALGESIC ACTIVITIES OF DITHIENYLBUTENYLAMINES (I, $R^1$=Me) IN THE RABBIT, ONE HOUR AFTER SUBCUTANEOUS INJECTION

Thirty rabbits used for each compound

| Amino Group | Activity Ratio, Morphine Sulphate=1·0 | |
|---|---|---|
| | Mean | Limits of Error, p=0·95 |
| Dimethylamino .. | 0·78 | 0·38–1·6 |
| Diethylamino .. | 0·96 | 0·48–1·9 |
| Piperidino .. .. | 0·95 | 0·49–1·8 |

*In dogs* the ratios of the analgesic activities of the thienyl compounds to that of morphine are very much smaller than those found in rodents. Table III shows the results obtained in tests using two or more doses of each compound.

TABLE III

ANALGESIA IN DOGS INJECTED SUBCUTANEOUSLY

Activities relative to morphine sulphate calculated from (*a*) proportion of dogs showing a 100% rise in pain threshold and (*b*) the duration of this rise. Limits of error for p=0·95 in brackets

| Compound | No. of Dogs Used | Activity Ratio | |
|---|---|---|---|
| | | (*a*) From Proportion | (*b*) From Duration |
| Dithienylbutenylamines: | | | |
| Dimethylamino- | 20 | 0·15 (0·08–0·27) | 0·13 (0·07–0·24) |
| Ethylmethylamino- | 22 | 0·31 (0·18–0·54) | 0·38 (0·20–0·72) |
| Diethylamino- .. | 41 | 0·19 (0·12–0·31) | 0·23 (0·13–0·39) |
| Pyrrolidino- .. | 22 | 0·11 (0·06–0·21) | 0·10 (0·05–0·19) |
| Piperidino- .. | 18 | 0·12 (0·07–0·20) | 0·077 (0·04–0·15) |
| Pethidine hydrochloride .. .. | 20 | 0·074 (0·044–0·13) | 0·05 (0·026–0·097) |
| Morphine sulphate | 33 | [1·0] | [1·0] |

The slopes of the regression of the probit of animals showing a 100% elevation in threshold on log-dose were homogeneous (p=0.3) for all drugs, the overall weighted mean value being $3.34 \pm 0.47$. This level of analgesia was given in 50% of dogs by 1.04 mg./kg. of morphine sulphate (limits, p=0.95, 0.7–1.5). The slopes relating log-dose and the duration of effect in hours were also homogeneous (p=0.3) with a weighted mean value of $4.15 \pm 0.54$, and the dose of morphine sulphate required to elevate the pain threshold by 100% for one hour was 1.4 mg./kg. (0.96–2.2). The common slopes were used in estimating the relative activities of the compounds in the table.

The p95 limits are wider in the dog tests than in the rat tests, and this obscures the differentiation.

However, although relative to morphine the thienyl compounds are less active in dogs than in rodents, relative to one another their activities are about the same. The ethylmethylamino compound is the most active of the thienyl analgesics in both rats and dogs. Trial in man (Keele, 1952) indicates that the results in the dog are much closer to the relative potencies in man than those in the rat and rabbit.

The observations that the slopes of the regression lines relating dose and the duration of analgesia in dogs are homogeneous for all the drugs, and that their relative activities are the same when assessed by duration of effect as by the quantal method, indicate that there are no significant differences between their durations of effect.

Higher levels of analgesia were attainable with the thienyl analgesics than with very large doses of morphine sulphate. Doses of 20–40 mg./kg. of the ethylmethylamino or diethylamino compounds, for example, made dogs insensitive to mechanical stimuli (the corneal reflex remained intact).

Only the diethylamino compound has been administered intravenously; 1–5 mg./kg. rapidly caused narcosis lasting five minutes to one hour depending on the dose, followed by a period of reduced sensitivity to pain. Much smaller amounts were required to cause the same level of analgesia by this route than by subcutaneous injection, and the action was more rapid in onset and of shorter duration.

*In cats* the ethylmethylamino and diethylamino compounds given subcutaneously in doses of 5 to 20 mg./kg. caused analgesia comparable with that due to 0.5 to 2 mg./kg. of morphine. They also produced excitation and mydriasis, but in contrast to morphine the thienyl compounds did not cause vomiting.

*Toxic Actions in Mice and Rats.*—Several tests in which groups of 10 mice were injected by various routes with doses separated by an interval ratio of 1.5 provided the LD50 estimates shown in Table IV. In the mouse the thienyl compounds cause analgesia, hyperexcitability, mydriasis, and erection of the tail. Respiratory depression was the primary cause of death with subcutaneous administration, but a cardiovascular component may be equally if not more important with intravenous injection.

The subcutaneous toxicity of each of the five thienyl analgesics was determined concurrently with that of amidone in 50–90 g. rats. Table IV shows the LD50 and the toxicity relative to that of amidone in the rat for each compound. The latter estimates have varied less between repeat

TABLE IV

THE TOXICITIES OF THE DITHIENYLBUTENYLAMINES (I, $R^1$=Me) IN MICE AND RATS

The limits of error, p=0·95, are shown in brackets

| Amino Group | LD50 in Mice | | Rats (Subcutaneous) | |
|---|---|---|---|---|
| | Intravenous mg./kg. | Subcutaneous mg./kg. | LD50 mg./kg. | Relative Toxicity Amidone =1·0 |
| Dimethylamino- .. | 16 (13–18) | 100 (85–120) | 170 (140–220) | 0·42 (0·38–0·47) |
| Ethylmethylamino- | 17 (14–20) | 94 (61–140) | 63 (41–97) | 0·58 (0·31–1·1) |
| Diethylamino- .. | 16 (14–18) | 81 (72–92) | 45 (31–64) | 0·81 (0·46–1·4) |
| Pyrrolidino- .. | 25 (22–29) | 120 (85–170) | 92 (61–140) | 0·40 (0·22–0·73) |
| Piperidino- .. | 15 (12–17) | 120 (100–140) | 95 (77–120) | 0·75 (0·67–0·83) |

tests than have the absolute LD50 values. These various thienyl analgesics have 0.4 to 0.8 times the toxicity of amidone. As amidone in our rats has 2.3 times the potency of morphine in the rat analgesic tests (Green and Young, 1951) the analgesic potencies of the five compounds (Table I) range between 0.3 and 0.8 times that of amidone. Thus the toxicity: analgesic ratios are similar to that of amidone. Depending on the dose, the thienyl analgesics caused periods of excitement, respiratory depression, and somnolence. Respiratory depression seemed to be the primary cause of death with subcutaneous injections. Like pethidine, they do not cause rigidity of the abdominal muscles, whereas morphine and amidone do so.

There was no retardation in growth, change in the erythrocyte count, or damage to internal organs (the lungs, livers, spleens, and kidneys were examined histologically by Dr. D. J. Trevan) when subcutaneous doses of 6 mg./kg. of the dimethylamino, diethylamino, or piperidino compound, or 3 mg./kg. of the ethylmethylamino or 10 mg./kg. of the pyrrolidino compound, were injected daily in groups of six rats for two months.

*Toxic Actions in Dogs.*—The thienyl compounds not only resemble morphine in their analgesic effect but share many of its side-actions. On subcutaneous injection in dogs they cause respiratory depression, preceded occasionally by hyperpnoea, miosis, and sometimes slight salivation. They induce evacuation of the bowel in a small percentage of animals, particularly those with existing diarrhoea, but to a lesser extent than does morphine. Unlike morphine, they do not cause vomiting when administered subcutaneously. Further, the hypnotic action of these compounds is more pronounced, with smoother induction and recovery. The slight salivation and the miosis are eliminated by atropine.

Very large doses have been injected subcutaneously in a few dogs. Prolonged narcosis but no toxic actions were seen with amounts up to 100 mg./kg. of the dimethylamino or pyrrolidino compounds, or with 50 mg./kg. of the ethylmethylamino or diethylamino compounds. However, after 100 mg./kg. of the diethylamino compound the narcosis was accompanied first by tremors and then 70 minutes later by severe clonic convulsions. These were abolished by pentobarbitone sodium (30 mg./kg.) and subsequent recovery was uneventful. Convulsions, controlled by pentobarbitone sodium, have also followed the intravenous injection of 6 to 10 mg./kg. of this compound.

The side-actions of the thienyl analgesics were also examined in dogs under pentobarbitone sodium. Administered intravenously, they caused a sharp temporary fall in blood pressure, of 30 mm. Hg with 0.5 mg./kg. of the diethylamino compound, 50 mm. Hg with 1 mg./kg. of the dimethylamino and piperidino compounds, or with 0.1 mg./kg. of the ethylmethylamino, and 85 mm. Hg with 1 mg./kg. of the pyrrolidino compound. They also caused brief apnoea followed by recovery within 15 to 30 min.; the effect was of shorter duration than that of morphine or amidone at these doses. In experiments in which the tone of the small intestine was recorded with balloons at pressures of 8–15 cm. of water all the thienyl compounds caused intestinal spasm. The minimal effective doses were 0.02 to 0.07 mg./kg. of the dimethylamino, 0.02 mg./kg. of the ethylmethylamino, and 0.03 mg./kg. of the diethylamino compound. Morphine had an effect of equal magnitude at doses varying between 0.0005 and 0.005 mg./kg.

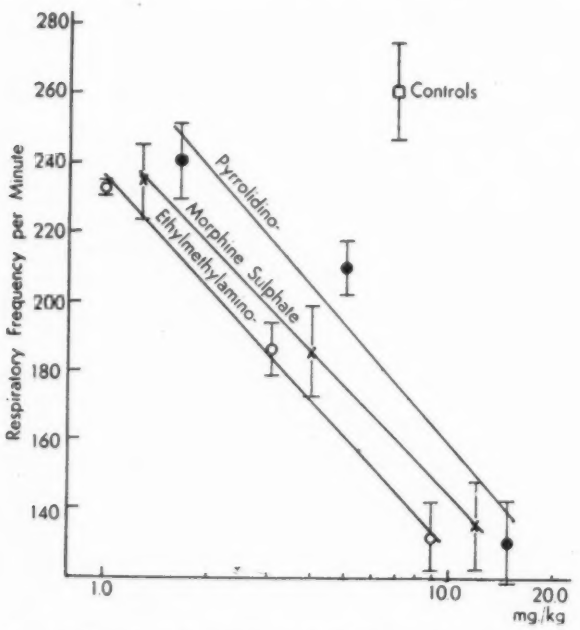



FIG. 2.—Frequency of respiration of rats thirty minutes after subcutaneous injection of dithienylbutenylamines or morphine sulphate. Means for groups of twenty rats with standard errors represented by vertical lines. The nature of the $NR^2R^3$ group is indicated on the graph.

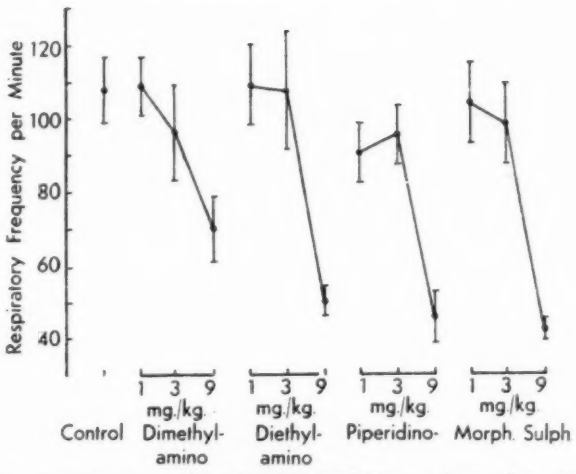



FIG. 3.—Frequency of respiration of rabbits one hour after subcutaneous injection of dithienylbutenylamines (I, $R^1$ = Me) or morphine sulphate. Means for groups of ten rabbits with standard errors represented by vertical lines. The nature of the $NR^2R^3$ group is indicated below the abscissae.

Administered in gelatine capsules by mouth, doses of 200 mg. of each of the five butenylamines caused vomiting in 50–75% of dogs, usually within 6–30 min., and effected only slight or moderate increases in pain threshold. As an emetic action has not been observed when administered by other routes it seems likely that these drugs have an irritant action on the stomach.

*Respiratory Depression.*—The mean respiratory frequencies of groups of 20 rats 30 min. after subcutaneous injection are plotted against the dose of the ethylmethylamino and pyrrolidino butenylamines and of morphine in Fig. 2. Deviations from linearity and parallelism were not significant and it was estimated from this data that the ethylmethylamino and pyrrolidino compounds were respectively about 1.3 and 0.7 times as depressant as morphine sulphate. These ratios do not differ significantly from the analgesic ratios in Table I.

*In rabbits* the magnitude of the respiratory depression caused by the dimethylamino, diethylamino, or piperidino compounds did not differ significantly from that with the same doses of morphine sulphate, whether the effect was estimated from changes in frequency in animals excited to some extent by handling (Fig. 3) or from changes in minute-volume in rabbits at rest

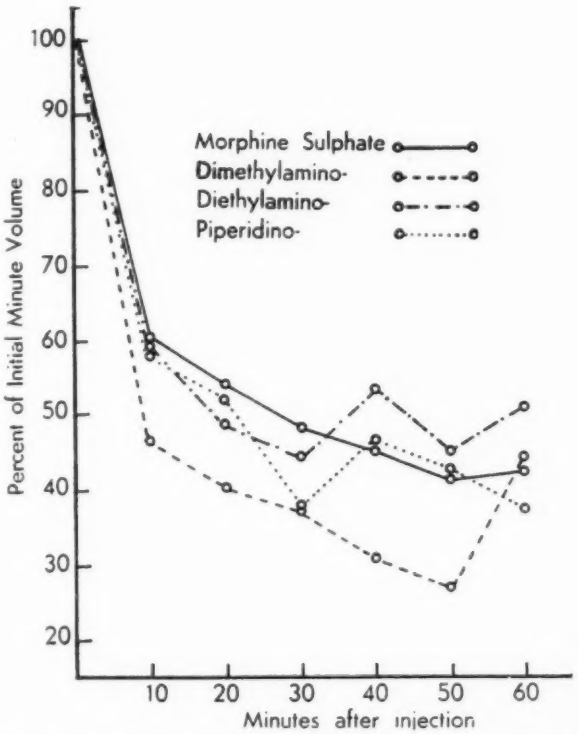



FIG. 4.—The respiratory minute-volume in rabbits as a percentage of initial at intervals following the subcutaneous injection of 2 mg./kg. of dithienylbutenylamines or morphine sulphate. Means for groups of four rabbits. The nature of the $NR^2R^3$ is indicated on the graph.

(Fig. 4). The depression in minute-volume in rabbits at rest was due mainly, though not entirely, to a reduction in frequency. It was associated with hypnosis, since with this dose (2 mg./kg.), and to a lesser extent with higher dosages, the minute-volume could be largely restored by disturbing the animals.

*Temperature.*—The thienyl compounds, like morphine, cause a fall in rectal temperature after subcutaneous injection into rabbits at rest (Fig. 5). The temperature depressant activities of the dimethylamino, diethylamino, and piperidino derivatives, relative to that of morphine, approximate to their analgesic potencies in this species. A fall of temperature also occurred on injection of the ethylmethylamino and morpholino analogues. The times taken both for the onset of the maximum fall in temperature and for recovery from each of the thienyl compounds tended to be shorter than those for morphine.

TABLE V

THE ACTIVITIES OF THE OPTICAL ISOMERIDES OF DITHIENYLBUTENYLAMINES (I, $R^1$ = Me) AS PROPORTIONS OF THOSE OF THE RESPECTIVE RACEMIC MIXTURES

The limits of error, p=0·95, are given in brackets

| Amino Group | Isomer [α]$^{20}_{5461}$ | *In vitro* Antagonism of | | Mydriasis (Mouse) | Analgesia (Rat) |
|---|---|---|---|---|---|
| | | Carbachoi | Histamine | | |
| Dimethyl-amino | *dextro* +118° | 1·63 (1·03–2·56) | 1·41 (0·96–2·07) | 1·18 (1·13–1·23) | 1·65 (0·98–2·79) |
| | *laevo* −125° | 0·89 (0·56–1·39) | 0·60 (0·41–0·88) | Not examined | 0·27 (0·17–0·44) |
| Pyrroli-dino | *dextro* +110° | 1·13 (0·78–1·66) | 1·33 (0·92–1·91) | 1·24 (0·92–1·68) | 1·51 (1·20–1·91) |
| | *laevo* −109° | 1·02 (0·70–1·49) | 0·81 (0·60–1·10) | 0·70 (0·52–0·94) | 0·64 (0·50–0·82) |

*Optical Isomers.*—The butenylamine hydrochlorides so far described are racemic mixtures. The component optical isomerides of the dimethylamino and pyrrolidino butenylamines have also been examined. The results of some comparative spasmolytic, antihistamine, mydriatic, and analgesic tests are shown in Table V, where the activity of each isomeride is given in terms of the activity of the respective racemic mixture. Qualitatively the pharmacological properties examined were similar, but the *dextro* isomeride of each compound was

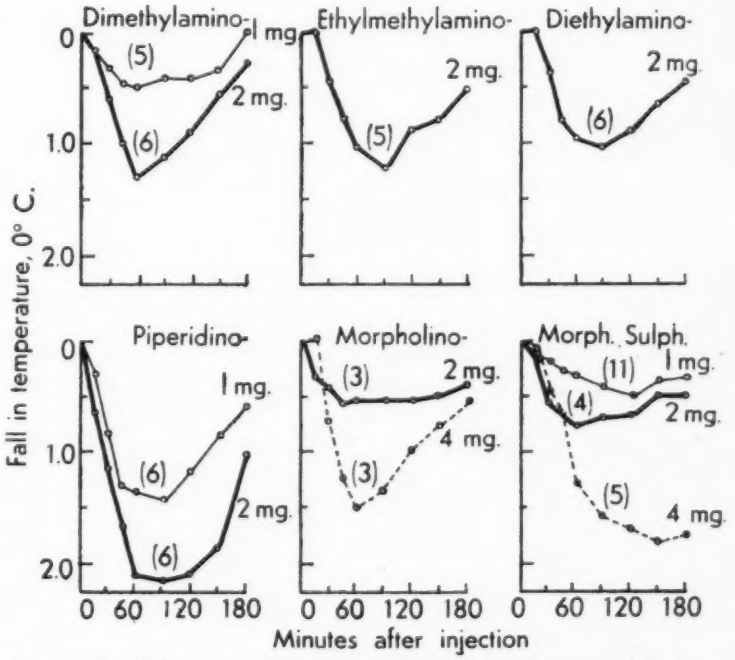



FIG. 5.—The fall in the rectal temperatures of rabbits after subcutaneous injection of dithienylbutenylamines and morphine sulphate. Doses in mg./kg. The number of rabbits in each group is shown in brackets. The nature of the $NR^2R^3$ group is indicated on the graph.

rather more active than the *laevo* form in most of the tests. The greatest difference observed was in analgesic potency, the *dextro* isomerides of the dimethylamino and pyrrolidino compounds being respectively about six and three times more active. This is in contrast with the finding that the *laevo* isomer is more active than the *dextro* in both amidone (Thorp, Walton, and Ofner, 1947) and in dromoran (Randall and Lehmann, 1950). In a comparative toxicity test where the compounds were injected intravenously in groups of ten mice, there was no significant difference between the toxicities of the pyrrolidino isomers. Estimates of the LD50 of the racemic, *dextro*, and *laevo* isomers were 25 mg./kg. (limits, p=0.95, 22–29), 23 mg./kg. (21–26), and 25 mg./kg. (21–31) respectively. Both the *d* and *l* isomerides of the pyrrolidino compound caused corneal anaesthesia after application of 0.5% solutions to rabbits' eyes.

## Discussion

There is a striking resemblance between the action of these dithienylbutenylamines and that of morphine. The slopes of the regression lines relating dose and increase in pain threshold in the rat and rabbit are similar to that of morphine, and similar high levels of analgesia may be attained. The relative analgesic, respiratory, and temperature effects parallel one another and like those of morphine are antagonized by N-allylnormorphine (Green and White, 1952, unpublished findings). Although the behaviour pattern of the mouse, rat, rabbit, cat, and dog injected with these compounds is similar to that after morphine, there are some differences. For example, the ethylmethylamino and pyrrolidino derivatives have a greater effect, relative to morphine, on the "flight" reaction to pressure on the tail of the rat than on the reflex removal of the tail from a source of heat stimulation. Unlike morphine and amidone they do not cause rigidity of the abdomen in rats. Further, these compounds do not cause vomiting on subcutaneous injection in the dog or in the cat. Experiments in anaesthetized dogs and in rats given charcoal meals (Green, unpublished) indicate that in comparable analgesic doses these compounds have a smaller effect than morphine on intestinal activity. A further difference from morphine is shown by the relative activities in different species, since in the rat and the rabbit several members have approximately the same analgesic activity as morphine but are only 0.1 to 0.4 times as potent in the dog and cat.

The dithienylbutenylamines have been tested in man by Keele (1952), who found that they were intermediate in activity between morphine and pethidine. Hence it seems that their relative activities in man and dog are similar. The species differences in sensitivity to analgesics might be due to the species differing in their modes of appreciation and response to pain stimuli ; alternatively the thienyl compounds might act at a different site from morphine. Another possibility now being explored is that the thienyl compounds, which are somewhat unstable in solution, may be more readily inactivated or excreted in man, the dog, and cat than in the rabbit and rat.

Many of the differences from morphine displayed by these thienyl compounds are shared by pethidine. For example, relative to morphine, pethidine exhibits an activity by the pressure test in the rat which is relatively higher than that by the radiant heat test (Green and Young, 1951). Pethidine does not cause rigidity of the abdomen ; it has a smaller effect than morphine on intestinal propulsion in the rat (Karr, 1947) and the dog (Vaughan Williams and Streeten, 1950). It is only about a fifth as active in man and the dog as it is in the rat. In these respects the actions of amidone more closely resemble those of morphine. It is therefore possible that the thienyl compounds and pethidine have a similar mode of analgesic action, which is in some respects different from that of morphine and amidone.

## Summary

1. Powerful analgesic properties and some atropine-like, antihistamine, and local anaesthetic properties have been found in a series of 3:3-dithienylalkenylamines.

2. 3-Dimethylamino, 3-ethylmethylamino, 3-diethylamino, 3-pyrrolidino, and 3-piperidino-1:1-di-2′-thienylbut-1-ene hydrochloride show activities varying between 0.6 and 1.7 times that of morphine sulphate in rats and rabbits, and between 0.1 and 0.4 times that of morphine in dogs and cats. The ethylmethylamino compound is the most active. Compared with morphine, their respiratory and temperature depressant activities are proportional to their analgesic potencies, but they seem to have less effect on the bowel. In this and other respects they and pethidine have a similar type of action, differing from that of morphine and amidone.

3. The *dextro* isomerides of the dimethylamino and pyrrolidino compounds exhibited greater analgesic, antihistamine, and atropine-like properties than the *laevo* isomerides.

The author is indebted to Drs. D. W. Adamson, J. W. Trevan, and A. C. White and Mr. P. A. Young for much useful advice, and to Messrs. J. B. Attlesey and J. A. Calnan and Mesdames I. Carter, E. D. R. Dann, and E. Stacey for valuable technical assistance.

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*Brit. J. Pharmacol.* (1953), **8**, 10.

# MYDRIASIS IN THE MOUSE: A QUANTITATIVE METHOD OF ESTIMATING PARASYMPATHETIC GANGLION BLOCK

BY

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It has been suggested that pentamethonium and hexamethonium differ in their effects on different ganglia (Paton, 1951), and it is desirable that their relative activities should be determined on several autonomic ganglia. Whereas determinations on the cat's superior cervical ganglion by the nictitating membrane preparation can be made fairly accurately, methods for measuring inhibitory activity on parasympathetic ganglia, for example, by inhibition of the peristaltic reflex of the guinea-pig or rabbit ileum (Feldberg and Lin, 1949), are subject to wider variations.

As a method which would give a reliable estimate of potency on the parasympathetic system, blockage of the ciliary ganglia, producing mydriasis in the mouse, has been selected. The method, which was first described by Pulewka (1932), and used by Ing, Dawes, and Wajda (1945) for studying atropine-like substances, was found by Wien and Mason (1951) to be applicable to ganglion blocking drugs, but they showed that the log. dose-response line obtained with hexamethonium had a curvilinear component, whereas that for atropine was rectilinear.

In the present investigation, the log. dose-response line of hexamethonium has been examined in some detail and it has been found that part of this is sufficiently rectilinear to enable trial assays of this compound to be carried out. From these trial assays suitable conditions for performing assays on other compounds in terms of hexamethonium were found, and the results with a few such compounds are recorded. Although the species (mouse) upon which the present experiments have been performed is one which differs in many of its physiological responses from higher mammals, it has the advantage, from a statistical viewpoint, that large numbers of animals may readily be used in each experiment.

## METHODS

*Experimental.*—The experimental procedure was essentially the same as that described by Ing *et al.* (1945). Inbred albino mice of both sexes, from our own laboratory stock, were used once only. For the main investigation mice of mean body weight 16 g. (15.5 to 16.5) were selected. The mice were placed singly in glass jars upon a white table placed directly beneath a fluorescent strip light (about 4 feet above), in an otherwise dark room. The jars, arranged in groups of five, were covered by wire mesh and the animals were left under these conditions for at least half an hour before the experiment. The pupil was measured, by means of a graduated scale in the eye-piece of a dissecting microscope, in the approximately antero-posterior diameter. After initial readings the animals were injected intraperitoneally with the drug to be examined, the volume of injection being 0.01 ml./g. body weight. Dose levels were separated by a log. interval of 0.17609. Subsequent readings were made at ten minutes after the injection and, in some experiments, also at twenty and thirty minutes.

The compounds investigated were hexamethonium and pentamethonium dibromides; hexamethylene-bis(ethyldimethylammonium) dibromide and tetramethylene-bis(diethylmethylammonium) dibromide (Barber and Gaimster, 1952; Wien and Mason, 1951; Wien, Mason, Edge, and Langston, 1952); tetraethylammonium bromide; and nicotine acid tartrate.

*Statistical.*—Although a record of each mouse was kept, the results were analysed by taking the total difference in response of each group of five mice. These totals were subjected to variance analysis and estimates of potency and fiducial limits calculated according to Fieller's formulae (1940; 1944).

## RESULTS

### *Examination of the Dose-response Line for Hexamethonium*

In view of Wien and Mason's (1951) findings, it was necessary to establish the shape of the log. dose-response line. In their experiments two curves are shown (Exps. 1 and 2); the first of these had a significant quadratic component, the second a significant cubic component. An analysis of a curve, Fig. 1*a*, determined in the present investigation, using groups of five mice on seven different occasions giving thirty-five mice per dose level, was performed.

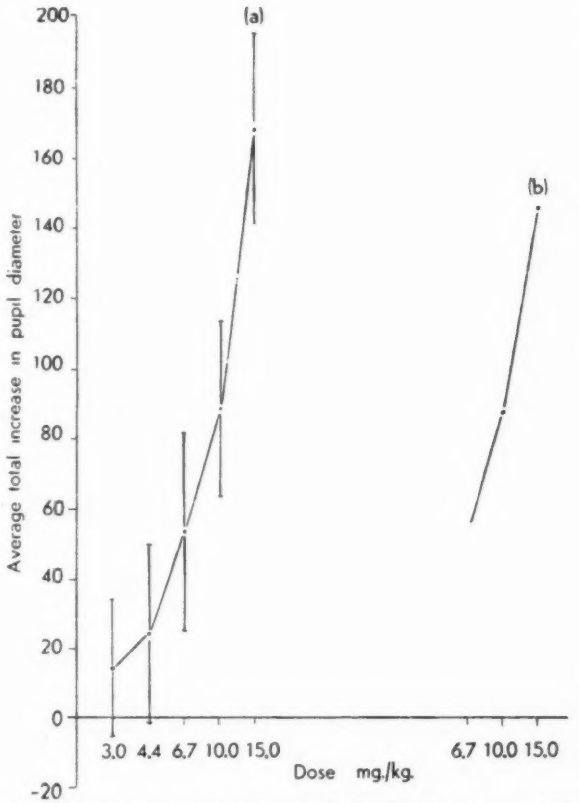


FIG. 1.—Dose-response lines for mydriatic effect in mice receiving graded doses of hexamethonium dibromide. (*a*) Each point represents the mean of seven groups, each of five mice: vertical lines show the standard deviation of each point. (*b*) Each point represents the mean of 27 groups, each of five mice.

An analysis of variance showed that the rectilinear and first degree curvilinear components were both very highly significant ($P<0.001$), although the variance ratio for the latter component was very much less than for the former. Neither the cubic nor quartic components were statistically significantly ($P>0.2$). In other words, the log. dose-response line is a smooth parabola. Converting to responses to log. units straightened the line, but this procedure was not an improvement because the standard deviations regressed from 1.03 to 0.07 from the lower to the upper points. It was found that correction by covariance analysis for the initial pupil diameter made no significant alteration in the standard deviation of the experiment as a whole ($P>0.2$), and the values of the mean squares were also practically unaffected: thus the conclusions drawn from the uncorrected variance analysis are in no way altered or modified. Inspection of Fig. 1*a*, however, shows that most of the curvature resides in the lower half of the curve and therefore an analysis of variance and covariance was made using only the top three dose-levels. The results revealed that the rectilinear component was very highly significant ($P<0.001$), whereas the first degree curvilinear component was still significant, although much less so ($P<0.05$). It was found that correction by covariance analysis for the initial pupil diameter made a highly significant improvement ($P<0.01$) in the standard deviation of the experiment as a whole, whereas all factors except rectilinearity, which was still very highly significant ($P<0.001$), became insignificant ($P>0.05$). In Fig. 1*b* is shown the log. dose-response line for hexamethonium dibromide, each point representing the mean response (not corrected for initial pupil diameter) of 27 groups of five mice each. This line has, in fact, a significant quadratic component ($P<0.05,>0.01$), the variance due to groups being insignificant ($P>0.05,\simeq 0.2$); the standard deviation is 24.21. However, in assays carried out on hexamethonium and other ganglionic blocking drugs reported later, in which only three or four groups of animals were used on each of three dose levels, deviations from parallelism and curvilinearity were not encountered, except for tetraethylammonium bromide. Thus for practical purposes this part of the log. dose-response line may be regarded as rectilinear and therefore suitable for carrying out valid potency estimates without correcting the results by covariance analysis. These findings are discussed below.

The extreme upper end of the log. dose-response line has not been studied so thoroughly because the pupil of the mouse can be made to dilate until its area is greater than the visible area of the cornea. With these larger diameters, it becomes necessary for the experimenter to move his own focus appreciably from one end of the scale to the other, and since it is impossible to hold the animal absolutely still, the estimation becomes more inaccurate with increase in pupil diameter.

Although it is not an unusual finding that the lower part of a log. dose-response line is not rectilinear, it was thought possible, in view of the rectilinear line obtained with atropine that hexamethonium was in fact effectively blocking not only the parasympathetic (ciliary) ganglion, controlling miosis, but also the sympathetic (superior cervical) ganglion, impulses from which can produce mydriasis. If this were so a relative miosis might be demonstrated by administering hexamethonium together with a maximal or near maximal dose of atropine sulphate, a method which has been successfully adopted by Grewal (1951) for examining anticholinesterases, muscarine-like compounds, and other miotics. In the

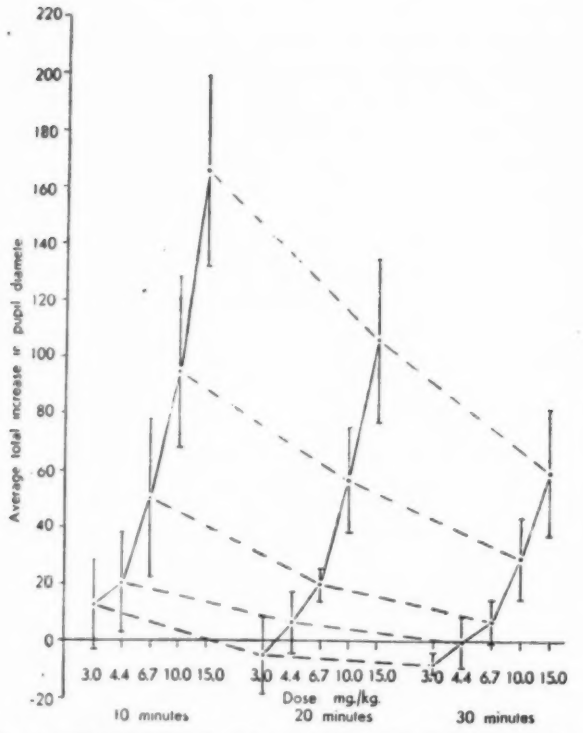


FIG. 2.—Dose-response lines for mydriatic effect in mice, showing response at 10, 20, and 30 minutes after injection, each point representing the mean of four groups of five mice each: vertical lines show the standard deviation of each point. All three curves determined on the same four groups of mice, corresponding doses being joined by broken lines.

presence of atropine, hexamethonium should be effective only on the sympathetic ganglion and should produce constriction of the pupil. This might provide a method for determining the potency of a ganglionic blocking drug on both divisions of the autonomic system, not only in the same species but on the same organ. The results of experiments in which doses of hexamethonium from 3.0 to 30.0 mg./kg. were used did not substantiate this hypothesis. This may be due to the fact that after both parasympathetic and sympathetic control has been inhibited by atropine and hexamethonium respectively, the pupil assumes a position of nearly maximum dilatation, which would make the observation of any changes difficult.

### *The Effect of Time, Groups, and Body Weights*

Three other factors have been investigated. Firstly, responses at varying times after injection are shown in Fig. 2; these graphs show that the variation about the mean for each dose is less when observed at twenty than at ten minutes. However, since Wien and Mason (1951) found a maximal response at ten minutes, this reading only was recorded in all subsequent work. When the top three dose levels only were used, the mydriatic activity at 20 minutes in terms of that at 10 minutes was 71.0% (58.5 to 83.1%) with $P=0.05$. Secondly, the variation between groups of mice was significant ($P<0.01$) only when these were tested on different days and not when tested on the same day (morning and afternoon), ($P>0.2$). This implies that an increase or decrease in the efficiency of the experimenter does not occur during any one day, and consequently eliminates the necessity for randomizing the order of injecting the doses. The third factor investigated was the possible effect of body weight upon the response obtained. It was found that there was no significant difference ($P>0.2$) in the response obtained by using animals of 16 or 20 g. body weight.

### *Assays of "Unknown" Solutions of Hexamethonium*

Assays of "unknown" solutions of hexamethonium dibromide have been carried out, and the analysis of variance of one such four-point assay is shown in Table I. In this particular assay the solution was estimated to have a potency of 129.6% of the standard solution with fiducial limits

TABLE I

VARIANCE ANALYSIS OF 4-POINT ASSAY OF STANDARD AND "UNKNOWN" SOLUTIONS OF HEXAMETHONIUM DIBROMIDE

| Variance Due to | Degrees of Freedom | Mean Squares | Variance Ratio | Significance |
|---|---|---|---|---|
| Groups .. | 3 | 2,549.90 | 4.25 | $P>0.05<0.2$ |
| Treatment: | | | | |
| (1) Standard and "unknown" | 1 | 5,148.06 | 8.57 | $P<0.05>0.01$ |
| (2) Slope .. | 1 | 12,600.06 | 20.98 | $P<0.01>0.001$ |
| (3) Departure from parallelism .. .. | 1 | 1,1[illegible]9.06 | 1.896 | $P>0.2$ |
| Error .. | 9 | $s^2=600.67$ | $s=24.51$ | |
| Total .. | 15 | | | |

TABLE II

ESTIMATED POTENCIES OF "UNKNOWN" SOLUTIONS OF HEXAMETHONIUM

| Expt. No. | No. of Groups (5 mice each) | Estimated Potency (%) | Fiducial Limits ($P=0.05$) (%) | Actual Potency (%) |
|---|---|---|---|---|
| 1 | 4 | 129.6 | 105.9–187.6 | 120.0 |
| 2 | 3 | 122.3* | 99.8–171.9 | 112.5 |
| 3 | 3 | 77.6 | 52.4– 95.7 | 75.0 |
| 4 | 3 | 56.2† | 24.1– 74.8 | 50.0 |
| 5‡ | 4 | 101.0 | 90.2–113.3 | 100.0 |
| | 3 | 99.4 | 83.1–118.9 | |
| | 2 | 110.0 | 83.0–151.2 | |

* Not significantly different from standard. † Difference very highly significant ($P<0.001$). ‡ Six-point assay.

(P=0.05) of 105.9 to 187.6% (Table II); the true value was 120%. The values obtained with other "unknown" solutions are shown in Table II. The last of these (5) was a six-point assay and shows that increasing the number of dose levels increases the accuracy of the potency estimate, whereas estimates obtained on increasing numbers of groups reduces the fiducial limits of the estimate.

TABLE III

MYDRIATIC ACTIVITY OF GANGLIONIC BLOCKING DRUGS COMPARED WITH HEXAMETHONIUM DIBROMIDE (=100)

| Compound | Mydriatic Activity | Fiducial Limits |
|---|---|---|
| Tetraethylammonium bromide .. | 33.2* | 27.2–43.8 |
| Pentamethonium dibromide .. | 28.4 | 24.0–34.3 |
| Hexamethylene-bis-(ethyldimethyl-ammonium) dibromide dihydrate | 301.4 | 257.4–349.2 |
| Tetramethylene-bis-(diethylmethyl-ammonium) dibromide .. .. | 289.7 | 250.9–343.3 |

* In this assay there was an opposed curvature.

*Assays of Other Ganglion Blocking Drugs*

The compounds examined are listed in Table III, the results being expressed in terms of the activity of hexamethonium dibromide, which was used as a standard throughout. For each compound the estimation of potency and fiducial limits was made by carrying out a six-point assay repeated on four different occasions, giving twenty animals on each of three dose-levels. In only one of these assays, namely that in which tetraethylammonium bromide was compared with hexamethonium dibromide, was there a significant deviation from parallelism ($P<0.01, \geqslant 0.001$), and in this assay only there was a significant opposed curvature ($P<0.05, \geqslant 0.01$). Tetraethylammonium bromide was one-third as active as hexamethonium dibromide although its ganglionic blocking effect is known to be complicated by other effects which might account for the opposed curvature of the regression lines. In all the other assays, the log. dose-response lines were without significant deviation from parallelism and without combined or opposed curvature ($P>0.05$). It will be seen from Table III that the estimated potency of pentamethonium dibromide was 28.4%, a figure which may be compared with 33.3% found by Paton and Zaimis (1949) on the rabbit ileum, and 75.0% found by Wien, Mason, Edge, and Langston (1952) on the guinea-pig ileum. The bis-ethyldimethyl-ammonium homologue of hexamethonium dibromide was 301.4% as potent as hexamethonium dibromide in its mydriatic effect, which may be compared with Wien and Mason's (1951) figure of 200% on the guinea-pig ileum. The compound, tetramethylene-bis-(diethylmethylammonium) dibromide (Wien *et al.*, 1952) possessed a mydriatic activity of 289.7%, whereas Wien *et al.* found it to have a ganglion blocking activity of only 25% on the guinea-pig ileum and 50% on the cardiac vagus of the cat.

It was not possible to estimate nicotine acid tartrate, since it was found that maximum dilatation of the pupil occurred at two to four minutes after injection, and that in order to obtain mydriasis at 10 minutes the doses required were within the toxic range.

## DISCUSSION

The log. dose-response line for mydriasis in the mouse produced by hexamethonium dibromide, unlike that for atropine sulphate (Ing *et al.*, 1945; Wien and Mason, 1951), is parabolic. The reason for this still remains obscure, although Wien and Mason (1951) have suggested it is probably due to a different mode of action. For a certain range of dose levels, i.e., from 6.6 to 15.0 mg./kg. of hexamethonium dibromide, the curvature arises from the variation encountered in the initial pupil diameters. However, from a statistical viewpoint, provided that the two log. dose-response lines in an assay are parallel the curvature does not invalidate an estimate of potency, since the two lines are separated by a constant horizontal distance. Moreover, in the assays performed, with the exception of that in which tetraethylammonium bromide, which is known to have mixed pharmacological actions, was compared with hexamethonium dibromide, neither deviations from parallelism nor curvature were encountered. It would thus seem that one may safely make an estimate of potency, with its fiducial limits, without correcting for initial pupil diameter, although if this correction was found to reduce the standard deviation of the experiment appreciably, the fiducial limits of the estimate might also be reduced. Moreover, converting the responses to log. units had little effect on the potency estimate and consistently increased the fiducial limits.

The result obtained with tetramethylene-bis-(diethylmethylammonium) dibromide seems particularly worthy of comment, since by this method its activity, in terms of that of hexamethonium, is very different from that found when tested on other preparations. This may represent a true differential action of this compound, not only with respect to the parasympathetic as opposed to the sympathetic system, but also within the parasympathetic system itself; or it may merely represent a difference due to species variation.

## Summary

1. It has been shown that the log. dose-response line obtained with hexamethonium dibromide in producing mydriasis in the mouse can be used for quantitative comparisons of similar compounds, since part of this line can be regarded as rectilinear. The variation encountered between groups of mice receiving the same dosage is greater when performed on different days than on the same day.

2. Although the final regression line obtained with hexamethonium dibromide has a slight curvature, assays performed with " unknown " solutions of hexamethonium and other closely related drugs are without curvature and do not deviate from parallelism. By using five mice per dose-level in a six-point assay, repeated on four different occasions, a reliable estimate of potency with fairly close fiducial limits may be obtained.

3. The mydriatic activities of the following compounds have been compared with hexamethonium dibromide: (*a*) tetraethylammonium bromide was about one-third as active, although this result may have been partly influenced by a direct stimulating action; (*b*) pentamethonium dibromide was about one-quarter as active; (*c*) the bis-ethyldimethylammonium homologue of hexamethonium and the bis-diethylmethyl homologue of tetramethonium, were both about three times more potent. These results were discussed in the light of the different effects of these compounds on other ganglia.

4. It was not possible to estimate nicotine acid tartrate by this method, mainly due to the very rapid onset and decline in its action.


I wish to express my thanks to Dr. R. Wien for suggesting this work to me, and to the Directors of May & Baker, Ltd., for allowing it to be published.

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*Brit. J. Pharmacol.* (1953), **8**, 15.

# EFFECT OF RESPIRATORY INHIBITION ON *SCHISTOSOMA MANSONI**

BY

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The observed survival of adult *Schistosoma mansoni in vivo* under conditions producing a marked reduction of respiration of this parasite has been reported previously from this laboratory (Bueding, Peters, and Welch, 1947). Mature schistosomes removed from the portal venous circulation of rabbits (which had previously been given repeated daily intravenous injections of a cyanine dye) exhibited a normal rate of glycolytic activity, although their oxygen consumption was greatly diminished. No evidence of a concurrent chemotherapeutic action of the dye was observed, and no differences in the appearance, motility or distribution of the parasites were detectable in comparison with parasites from untreated animals.

This observation has been extended by the experiments described below. One of these shows that repeated injections of the same compound into hamsters infected with *S. mansoni* seven weeks previously, produced no observable effect on the mature worms other than a depression of respiration. However, other experiments demonstrated that significant changes in the course of infection were associated with this respiratory depression, when treatment was begun at an earlier stage of development of the parasite. It is this latter observation and its implications which is believed to deserve publication.

## METHODS

The cyanine dye, 1′-ethyl-3:6-dimethy-2-phenyl-4-pyrimido-2′-cyanine chloride, whose structural formula appears elsewhere (Welch *et al.*, 1947; Peters *et al.*, 1949; Bueding, 1949a) was administered intraperitoneally as a 0.125% (w/v) solution in 0.85% (w/v) NaCl.

Young Syrian hamsters, weighing 85 to 120 g., were exposed to freshly shed cercariae from laboratory-infected snails (*Australorbis glabratus*, Puerto Rican strain). Infection was accomplished in Exps. 1 and 2 (Tables I and IIA) by intraperitoneal injection of aqueous suspensions of 160 to 180 cercariae. The alternative procedure used in Exps. 3 and 4 (Tables IIB and III)—namely, immersions of the animals in similar suspensions—ultimately led to heavier infections with adult schistosomes.

Faecal pellets from the individually housed animals were collected on clean moist blotting-paper placed under the wire-mesh cage flooring. Single pellets were transferred to glass slides, mixed with several drops of distilled water, covered with a glass slip and examined microscopically for ova (100-fold magnification). Ova were found in untreated animals six to eight weeks after infection. Whenever repeated examination failed to reveal the presence of ova up to the time of sacrifice, mucosal scrapings from the large bowel were examined microscopically *post mortem*.

Animals were usually sacrificed for necropsy 24 hours after the last injection. Of the remainder, those found moribund as a result of the infection were sacrificed immediately; those found dead were discarded. The flukes were removed from the portal and mesenteric veins with dissecting needles, and were transferred, after counting, to a salt solution containing 200 mg. glucose per 100 ml. (Bueding, 1950). Lots of eight to ten worm pairs then were transferred to 0.8 ml. volumes of a similar solution contained in small Warburg vessels (total volume of vessels: 5 to 5.5 ml.). The oxygen uptake was measured manometrically over a period of two hours. Schistosomes from untreated animals served as controls. Comparison of the oxygen uptake in two, and at times three, vessels containing flukes from a single hamster revealed a maximum variation of only 8%.

Other worm pairs from treated and untreated animals were fixed in a solution containing 4% (w/v) formaldehyde, 50% alcohol (v/v), and 2% (v/v) acetic acid (Landowsky's mixture) by vigorous shaking in small vials. The whole worms were subsequently stained with Mayer's carmalum and were examined microscopically for morphological changes. In the

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†Markle Scholar in Medical Science.

female worms particular attention was given to the development of the ovary and vitelline glands, the presence of sperm in the oviduct, and the presence of an egg in the oötype or uterus. Male worms were examined especially for testicular development and for the presence of sperm in the seminal vesicles.

Sections of the livers of treated and untreated animals were also removed at necropsy. Several small weighed fractions (8 to 14 mg.) of each of these were immediately pressed between glass slides and examined microscopically for the presence of schistosome ova. Quantitative results of such examinations are expressed as the number of ova per mg. of tissue (wet weight). In addition, livers of treated and untreated animals were fixed by immersion in a 4% (w/v) solution of formaldehyde in saline. Histological sections of these were examined microscopically for pathological changes.

Some of the animals in each experimental group served as controls and were not treated with the cyanine. They, and the schistosomes and livers removed from them, were subjected to procedures otherwise identical with those described.

Quantitative data were analysed statistically by standard methods (for example, Burn, 1930). Such data have been expressed in the Tables as means ± the standard error of the mean. Corresponding mean values for treated and untreated experimental groups were considered to be significantly different if a value greater than 2.5 resulted on dividing the differences between the two means by the square root of the sum of the squares of the standard errors.

## Results

No signs of a chemotherapeutic effect were detected in hamsters infected with *S. mansoni* when treatment with cyanine dye was deferred until seven weeks after infection. Characteristic ova, indicative of maturity of the worms, were present in the faeces of all animals at that time and persisted in approximately equal and undiminished numbers in both treated and untreated animals. Necropsy performed 24 hours after the last injection revealed no macroscopically visible differences between treated and untreated hamsters in the degree of focal fibrosis of the livers induced by deposition of and reaction to schistosome ova. As shown in Table I, there was no significant difference between the treated and untreated hamsters with respect to the average number of worms recovered per animal from the extrahepatic portal-venous system, or with respect to their distribution therein. Comparison likewise revealed no effect of therapy on the motility and appearance of the worms. In contrast, therapy with the cyanine dye produced a marked depression in the oxygen uptake of the schistosomes. As shown in Table I, the average rate of respiration of the worms removed from treated animals was less than 50% of that of worms removed from controls.

By contrast, when treatment with the cyanine dye was begun at an earlier interval, definite effects on the course of the infection were discernible. Tables II and III illustrate these effects under different experimental conditions.

Table I

THE EFFECT OF DELAYED TREATMENT WITH CYANINE DYE ON *SCHISTOSOMA MANSONI* IN HAMSTERS

Treatment begun seven weeks after cercarial infection when all animals were excreting ova; dose: 5.0 mg./kg. i.p. daily for four to eight doses, then 2.5 mg./kg. daily. No difference was observed between treated and untreated animals with respect to excretion of ova; gross pathological changes in liver of host; motility, distribution, degree of pairing, gross and microscopic appearance of worms

| Group | Total Dose mg./kg. | Duration of Therapy (Days) | Necropsy—24 Hr. After Last Dose | | |
|---|---|---|---|---|---|
| | | | Days after Infection | No. of Worm Pairs in Portal and Mes. Veins | $Qo_2$ |
| Treated (11) .. | 20–55 | 4–15 | 53–64 | 9±1.0 | 4.1±0.2 |
| Untreated (12) .. | 0 | 0 | 53–64 | 11±1.4 | 10.0±0.3 |

Table II

THE EFFECT OF EARLY TREATMENT WITH CYANINE DYE, 3 MG./KG. I.P. DAILY, ON *SCHISTOSOMA MANSONI* IN HAMSTERS

| Treatment | Group | Appearance of Ova in Faeces (Days after Infection) | Necropsy | | | | |
|---|---|---|---|---|---|---|---|
| | | | Days after Infection | Hepatic Involvement | | No. of Worm Pairs in Portal and Mes. Veins | $Qo_2$ |
| | | | | Ova/mg. Wet Weight | Pathological Changes | | |
| A. From 27th day after cercarial infection | Treated (18) | 60±0.4 | 57–71 | 4.6±0.5 | 1+ to 2+ | 19±0.5 | 2.9±0.14 |
| | Untreated (16) | 50±1.25 | 41–56 | 16±1.0 | 3+ to 4+ | 31±1.6 | 8.5±0.35 |
| B. From 9th day after cercarial infection | Treated (22) | 58±0.5 | 52–64 | 2.7±0.5 | 1+ (14)<br>2+ (8) | 7±1 portal<br>6±0.8 mes. | 4.5±0.45 |
| | Untreated (13) | 47±0.4 | 54–66 | 7.8±1.0 | 2+ (3)<br>3+ (6)<br>4+ (4) | 6±0.9 portal<br>11±0.4 mes. | 6.7±0.33 |

In the experiment shown in Table IIA, therapy was begun approximately three weeks before ova appeared in the faeces of a group of untreated animals studied at the same time. The delay in excretion of ova, the decrease in the number of worms recoverable at necropsy, and the lesser degree of hepatic involvement in the treated animals were all highly significant statistically, and all indicate that the dye exerted a definite chemotherapeutic effect. The reason for the earlier necropsy of untreated animals was that their infection had reached a terminal stage, whereas treated animals remained healthy. Had treated animals been necropsied as early as untreated controls, the difference in the degree of infection would probably have been even more apparent. When the livers of five untreated hamsters were minced and suspended in water at 30° C., innumerable miracidia were produced by hatching of viable eggs. The low degree of viability of ova from worms of treated hamsters was illustrated by the fact that only two of eighteen livers examined yielded one miracidium each, and the remainder none. Histological sections of liver stained with haematoxylin-eosin revealed a significantly smaller degree of tissue reaction and tubercle formation around deposited ova in treated, as compared to untreated, animals. The depressant effect of the dye on the oxygen uptake of the worms was of the same order of magnitude as that which occurred in the first experiment (Table I).

TABLE III

THE EFFECT OF A SHORT PERIOD OF THERAPY WITH CYANINE DYE BEFORE MATURATION OF THE WORMS ON THE SUBSEQUENT COURSE OF SCHISTOSOMIASIS IN HAMSTERS

Treatment: 3 mg. of cyanine dye per kg., administered i.p. daily from 30th to 40th day (incl.) after cercarial infection.

| Group | Appearance of Ova in Faeces (Days after Infection) | Necropsy | | | | |
|---|---|---|---|---|---|---|
| | | Days after Infection | Hepatic Involvement | | No. of Worm Pairs in Portal and Mes. Veins | $Qo_2$ |
| | | | Ova/mg. of Liver (wet wt.) | Pathological Change | | |
| Treated (28) | 49–56 | 41–56 | 4.0±0.6 | ± (5)<br>1+ (9)<br>2+ (5)<br>3+ (9) | 51±2.8<br>24±1.6 portal<br>27±1.7 mes. | 5.8±0.3 |
| Untreated (18) | 48–56 | 41–56 | 7.2±0.9 | 1+ (2)<br>2+ (3)<br>3+ (9)<br>4+ (4) | 51±3.7<br>26±1.9 portal<br>31±2.4 mes. | 8.6±0.4 |

On the basis of these findings it seemed possible that treatment begun at an even earlier interval might exert a curative effect. Therefore, in another experiment (Table IIB) treatment was begun only nine days after infection, i.e. approximately five weeks before ova appeared in the faeces of untreated controls. The treated animals showed a significant delay in the excretion of ova and a significant decrease in the degree of hepatic involvement resulting from egg deposition. A cyanine-induced decrease in the viability of ova was again illustrated by the fact that none of the livers from 22 treated hamsters yielded miracidia, when minced and suspended in water, whereas miracidia appeared rapidly in suspensions from nine of 13 livers in the untreated group. However, none of these differences was greater than those seen in animals whose treatment began on the 27th day after infection. Furthermore, the reduction in the number of parasites recovered at necropsy was much less; it was statistically significant only with respect to the number found in the mesenteric veins, but not in the portal vein. An equally surprising finding was the fact that the cyanine dye in this experimental group produced a smaller, though significant, reduction in the oxygen uptake of the worms. This suggested the possibility that an alternative pathway for metabolism, less susceptible to inhibition by cyanine dyes, had been developed as a result of exposure to this agent at a very early and possibly highly adaptive stage of development of the parasite.

In the experiment shown in Table III, treatment was begun at an intermediate interval after infection, approximately the same as that of the experiment in Table IIA. Therapy was discontinued on the tenth day so that the total dose was within the range of that given at an advanced stage of infection in the first experiment (Table I). Necropsies were begun on the day following the last dose of drug. As shown in Table III, the oxygen consumption of worms from treated animals was significantly depressed. The less marked hepatic involvement in treated animals was indicated by the fact that pathological changes were less advanced and the egg content of the livers was statistically lower than that of livers from the untreated group. The differences in $Qo_2$ and hepatic egg content were considerably greater when only animals necropsied before ova appeared in the faeces were compared, i.e., animals sacrificed soon after cessation of therapy. This would suggest that worms which had been exposed to the dye recovered considerably at intervals further removed from the therapy period. Statistically, however, the differences were no greater than when all animals were considered, because of the small

number of animals in the early groups. Finally, the interval at which excretion of ova began and the number of worms recovered at necropsy were not affected by treatment.

Microscopic studies failed to reveal any morphological differences between schistosomes removed from treated and untreated animals in the four experiments described.

## Discussion

A high rate of aerobic metabolism is essential for some parasitic helminths. For example, the filarial nematode, *Litomosoides carinii*, survives for less than 12 hours in an atmosphere of nitrogen (Ross and Bueding, 1950), and reduction of its rate of respiration by the administration of cyanine dyes to its mammalian host results in the death of these worms (Welch *et al.*, 1947 ; Peters *et al.*, 1949).

*S. mansoni* is considerably more resistant to anaerobiosis and to inhibition of its oxygen uptake. It has been suggested (Bueding, 1949b) that this difference may be a quantitative rather than a qualitative one ; i.e., that this parasite has a much smaller requirement for respiratory metabolism, but, nevertheless, a very definite and ultimately essential one. Though it can survive *in vitro* in the absence of oxygen for five days, survival under aerobic conditions has been observed for as long as 16 days (Ross and Bueding, 1950). The present study shows that partial inhibition of the respiratory metabolism of *S. mansoni* in hamsters, by treatment of the host with a cyanine dye, had no noticeable effect on the worms when such therapy was of relatively short duration, and was deferred until after maturation of the worms to the egg laying stage (Table I). When therapy of comparable intensity (Table III) was begun shortly before maturation of the parasites, a mild chemotherapeutic effect was reflected in a slight inhibition of egg production. This chemotherapeutic effect was increased greatly when therapy was continued for a much longer period (Table IIA) ; under these circumstances egg production was much reduced and there was also a definite reduction in the number of worms recoverable from the portal venous system of the host. Though the criteria for appraisal of chemotherapeutic activity were not equally applicable to all stages of the infection, the results suggest that dependence on aerobic metabolism is greater just before maturation of the parasite. Finally, when intensive and prolonged therapy was begun at a still earlier stage of development of the parasites (Table IIB), the sensitivity of the worms to the cyanine dye was not as great, from either a chemotherapeutic or a metabolic standpoint, as it was when therapy was begun several weeks later (Tables II and III). It is possible that at the very early stage of development the worms may be capable of developing a pathway of respiratory metabolism which is not susceptible to inhibition by the cyanine dye.

The relatively low dependence of *S. mansoni* on oxidative metabolism indicates that a major portion of the energy derived for survival and reproduction of this organism originates from anaerobic reactions. This might explain its very high rate of glycolysis, which is not affected by oxidative metabolism (Bueding, 1950). It appears likely, therefore, that inhibitors of essential anaerobic reactions would hold forth greater promise as potential chemotherapeutic agents against *S. mansoni* than would compounds, such as the cynanine dyes, which affect its oxidative metabolism.

## Summary

1. Inhibition of the oxidative metabolism of *S. mansoni* by administration of a cyanine dye to infected hamsters is not associated with the dramatic chemotherapeutic effects observed in another parasitic worm, the filarial nematode, *L. carinii*.

2. When daily parenteral injections of the dye into infected hamsters are begun two weeks before maturation of *S. mansoni* and are continued until four to six weeks after maturation, a mild though definite chemotherapeutic effect is observed.

3. Shorter periods of therapy, or therapy begun earlier or later in the cause of the infection, were much less effective. This suggests that the dependence of the parasite on oxidative metabolism for survival is of a low order, and varies to some extent with the stage of development of its life cycle.

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# STUDIES ON THE PHARMACOLOGY OF SUCCINYLCHOLINE

BY

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Succinylcholine, a choline ester of succinic acid, is a short-acting neuromuscular relaxant. A review of its chemical, pharmacological, and clinical properties has been published elsewhere (Bourne, Collier, and Somers, 1952). Previous studies on the pharmacology of this compound have been largely confined to the dog and rabbit, and a closer study of its actions in the cat seemed desirable. Organe, Paton, and Zaimis (1949) showed that the actions of decamethonium in the cat are more closely related to those in man. Chemically, succinylcholine shows a close structural relationship to acetylcholine, and it was important to ensure, before clinical trial, that succinylcholine was free from muscarine-like actions.

## METHODS

Four cats were anaesthetized with chloralose (80 mg./kg.). Twitches of the soleus and tibialis anterior muscles, stimulated with supramaximal shocks applied to the peripheral end of the sciatic nerve in the thigh, were recorded with a flat spring myograph as described by Paton and Zaimis (1949). All injections were made intravenously and the doses recorded are those for succinylcholine chloride per kg. body weight. Salivary flow was recorded in three cats and two dogs, anaesthetized with chloralose, by a drop recorder (Gaddum and Kwiatkowski, 1938) after cannulation of Wharton's duct.

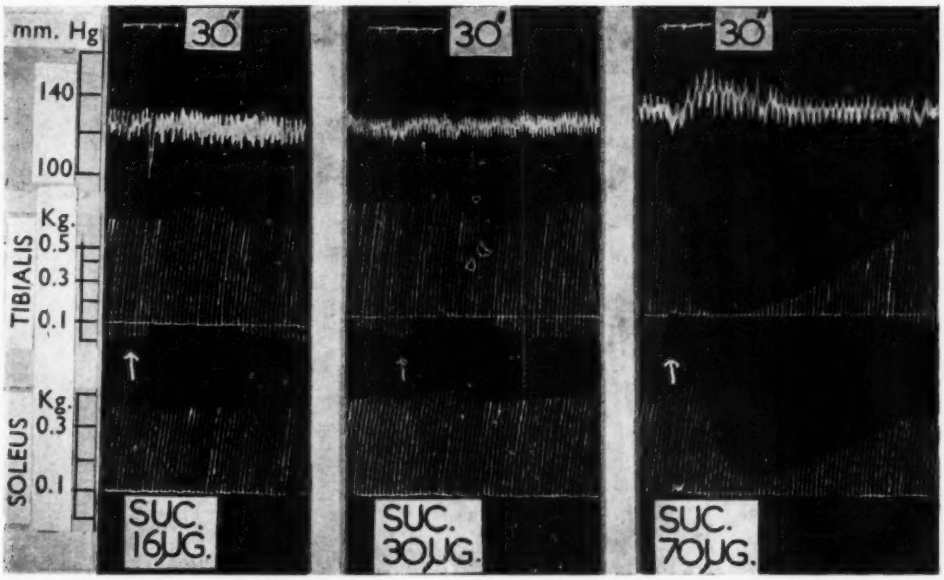


FIG. 1.—Cat, chloralose. Records of blood pressure (above), and of the contractions of tibialis anterior (middle) and soleus (below). Injections of succinylcholine 16, 30, and 70 $\mu$g. intravenously.

## RESULTS

*Neuromuscular Blocking Activity.*—Fig. 1 shows the effects on the soleus and tibialis muscles of increasing doses of succinylcholine. Like decamethonium, succinylcholine first caused an increase in the twitch tension and fasciculation of the muscles. This was followed by a brief period of paralysis, much shorter in duration than that caused by decamethonium. Doses could be repeated at

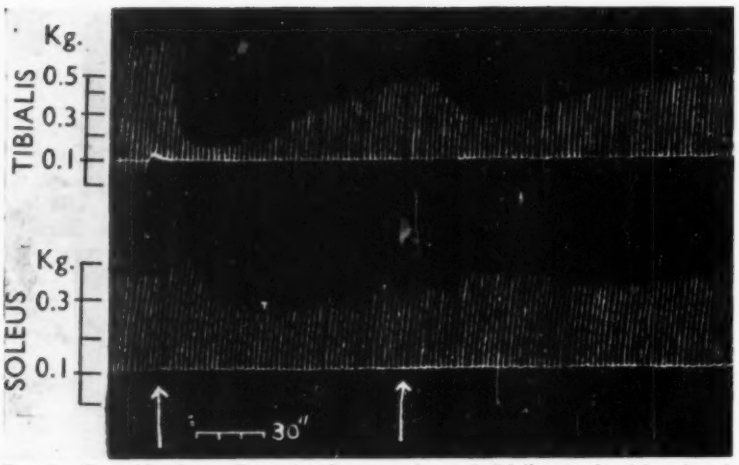


FIG. 2.—Cat, chloralose. Records of contractions of tibialis anterior (above) and soleus (below). At first arrow succinylcholine 50 μg. intravenously; at second arrow neostigmine 20 μg.

30-minute intervals without a cumulative action. Of particular interest was the difference in the sensitivities of the tibialis (" white " muscle) and soleus (" red " muscle), which was much less than that observed after administration of decamethonium (Paton and Zaimis, 1951). A dose of 70 μg. of succinylcholine, which did not completely paralyse tibialis, depressed the twitch tension of the soleus by 75% ; whereas a dose of 20 μg. of decamethonium, which caused a 75% depression of tibialis, actually increased the twitch tension of soleus.

*Effect of Neostigmine.*—Succinylcholine differs from decamethonium in being hydrolysed by cholinesterases (Glick, 1941 ; Bovet-Nitti, 1949). Thus neostigmine, which inhibits cholinesterases, prolongs the action of succinylcholine. Fig. 2 shows the effects of a dose of 20 μg. of neostigmine on tibialis and soleus. The tibialis was further depressed, while the soleus showed only an increase of twitch tension such as neostigmine causes on normal muscle.

*Muscarinic and Nicotinic Actions.*—The absence of muscarine-like actions was shown on the blood pressure and salivary flow of three anaesthetized cats and two dogs. Small doses of succinylcholine had no effect on the blood pressure, provided ventilation was adequate. In cats under artificial respiration 2 mg. of succinylcholine caused a slight rise in blood pressure and 10 mg. caused a large rise ; this was nicotinic in origin, since it was abolished by ganglionic blocking agents such as hexamethonium and tetraethylammonium chlorides (Fig. 3). Bovet, Bovet-Nitti, Guarino, Longo, and Fusco (1951) reported the hypertensive action of high doses of succinylcholine in the dog, but they also found that it caused a fall of blood pressure in small doses (Bovet, Bovet-Nitti, Guarino, Longo, Fusco, and Marotta, 1949). Succinylcholine did not increase salivary flow in cats or dogs, in contrast with the observations of Bovet *et al.* (1951) and Ginzel, Klupp, and Werner (1951), who reported an increase in the flow of saliva in the dog. Large doses of succinylcholine, 10 mg., slightly decreased the rate of salivary secretion (Fig. 4).

## DISCUSSION

Although succinylcholine resembles acetylcholine in chemical structure it shows only the nicotine-like actions and is free from muscarine-like actions. It would appear, therefore, that the larger molecule of succinylcholine is unable to combine with the muscarinic receptors. The neuromuscular blocking action of succinylcholine is similar to that of decamethonium, being preceded by a brief period of stimulation. Unlike decamethonium, however, succinylcholine is slowly hydrolysed by

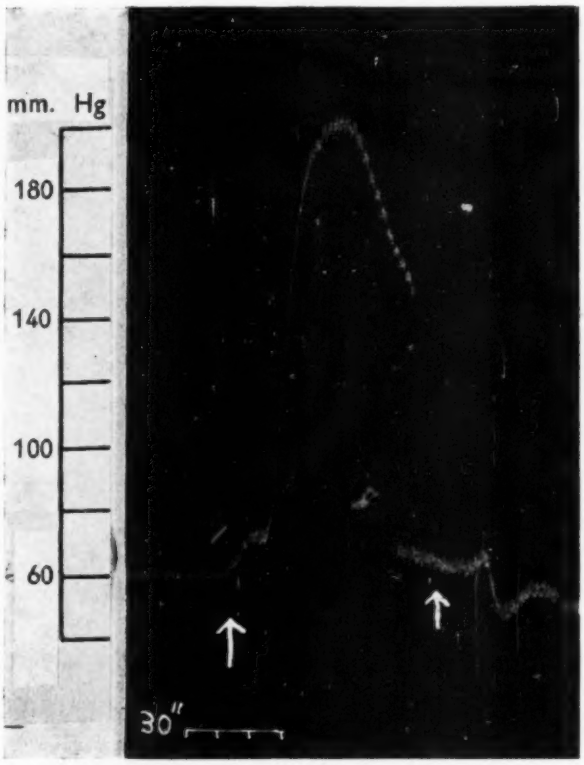


FIG. 3.—Cat, chloralose. Record of blood pressure. At first arrow succinylcholine 10 mg.; at second arrow succinylcholine 10 mg. after 20 mg. of tetraethylammonium.

cholinesterases and its action on the neuromuscular end-plate can be regarded as intermediate between that of acetylcholine and that of decamethonium. The difference in sensitivities of the "red" and "white" muscles is not as great as with decamethonium, which would suggest that succinylcholine will not have the sparing effect on the respiratory muscles claimed for decamethonium (Paton and Zaimis, 1951).

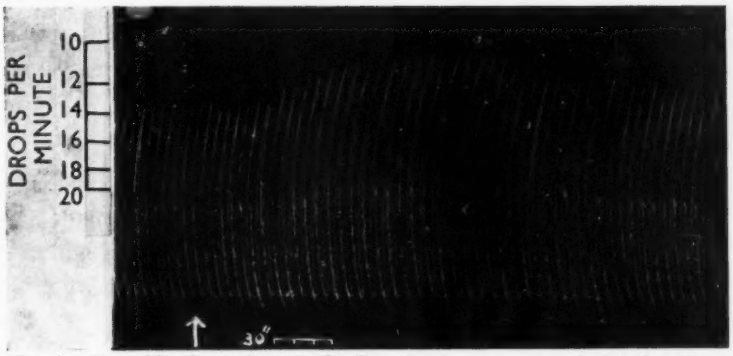


FIG. 4.— Dog, chloralose. Record of salivary flow. At arrow succinylcholine 10 mg. intravenously.


## SUMMARY

1. The neuromuscular blocking action of succinylcholine has been studied in the cat. Like decamethonium it first potentiates the indirectly excited maximal twitch, followed by a brief period of paralysis. There is not the same difference in sensitivities between soleus and tibialis as with decamethonium. Neostigmine potentiates and prolongs the paralysing action of succinylcholine.

2. Although closely related to acetylcholine in chemical structure, succinylcholine shows only the nicotinic actions and not the muscarinic actions of acetylcholine.


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# SOME MORPHINE-LIKE PROPERTIES OF (±)-*CYCLO*HEXYLOXY-*ALPHA*-PHENYLETHYLAMINES

BY

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Analgesic activity has been reported in a wide range of arylalkylamines, mainly derivatives of β-phenylethylamine (Fellows and Ullyot, 1951). The moderate degree of activity of *m*- and *p-cyclo*hexyloxy-α-phenylethylamine described below was of interest because of the close chemical resemblance to the antipyretic analgesic, phenacetin (*p*-ethoxyacetanilide). This activity might be related either to the antipyretics or to the opiates. Information on this point was sought in their general pharmacology, since it has been found empirically that analgesics, other than the antipyretics, show qualitative similarity over a range of pharmacological properties in spite of gross dissimilarity in chemical structure. For comparison, three closely related amines, *m*- and *p-iso*propyloxy-, and *p*-ethoxy-α-phenylethylamine, which are not analgesic, have been examined.

## RESULTS

The amines were given intraperitoneally (unless otherwise stated) as the hydrochlorides, which form stable neutral aqueous solutions.

*Acute Toxicity.*—Groups of 10 to 15 male albino mice were used at each dose level. Large doses induced convulsions of mixed type. The *cyclo*hexyl ethers caused violent, predominantly tonic convulsions with marked opisthotonos, after an initial phase of excitability with the Straub sign, but the *iso*propyl and ethyl ethers induced predominantly clonic convulsions with no Straub sign. Death was apparently due to respiratory failure. The LD50 and limits of confidence (Table I) were calculated as described by Emmens (1948).

*Analgesic Activity.*—The mean % elevation of reaction threshold, 30 minutes after injection, was assessed by Thorp's (1946) modification of the method of D'Amour and Smith (1941). Three groups of eight albino rats received the drug, saline, and morphine on alternate days. The *m*- and *p-cyclo*hexyl ethers gave linear log.-dose response curves, $y=200x-190$ and $y=138x-133$ respectively, where $y=\%$ elevation in reaction threshold and $x=$log. dose. Doses equivalent to morphine (4 mg./kg.) were respectively 11.5 and 13.5 mg./kg. The *iso*propyl and ethyl ethers were inactive at 20 mg./kg. Time-action curves (in a more sensitive strain of rats) showed that the maximal response to morphine was reached later than that to the *cyclo*hexyl ethers; the latter caused a sharp rise to a maximum in 10 to 20 minutes, followed by a rapid fall in reaction threshold. Averaged data from several curves are summarized in Table II. (The *m-cyclo*hexyl ether was not examined fully because of its pronounced tendency to produce local necrosis.) The *p-cyclo*hexyl ether was quite inactive subcutaneously, probably owing to failure of absorption, since the injection site slowly hardened and sloughed (cf. s.c. toxicity). Depressant activity was not measured, but it was noted in these tests that there was depression of spontaneous movement following the injection. This was especially marked after the *p-cyclo*hexyl ether. The depression outlasted anal-

TABLE I

ACUTE TOXICITIES OF ALKOXY α-PHENYLETHYLAMINE HYDROCHLORIDES IN MICE

| Structure R, R′ | | Hydrochloride | LD50 mg./kg. (Limits of confidence in parentheses) | | |
|---|---|---|---|---|---|
| R | R′ | m.p. | i.p. | i.v. | s.c. (abdomen) |
| H | O $C_6H_{11}$* | — | 117 (110–125) | 54 (50–60) | 388 (303–496) |
| H | O $C_3H_7$† | 86° | 132 (123–142) | — | — |
| O $C_6H_{11}$* | H | 180° | 170 (160–180) | 58 (49–69) | >800‡ |
| O $C_3H_7$† | H | 122° | 178 (170–187) | — | — |
| O $C_2H_5$ | H | 204° | 180 (168–193) | — | — |

* $C_6H_{11}$=*cyclo*hexyl. † $C_3H_7$=*iso*propyl. ‡ 20% mortality at 800 mg./kg.

TABLE II

COMPARISON OF THE MAXIMUM ANALGESIC ACTIVITY AND DURATION OF EFFECT, IN RATS, OF *p-CYCLO*HEXYLOXY-α-PHENYLETHYLAMINE HYDROCHLORIDE AND MORPHINE SULPHATE AFTER INTRAPERITONEAL INJECTION

| Drug | Dose mg./kg. | No. of Rats | Mean % Elevation of Reaction Threshold* | | Mean Peak Time* (min.) | Duration (approx.) (min.) |
|---|---|---|---|---|---|---|
| | | | At Peak | At 30 Min. | | |
| Morphine sulphate | 4 | 6 | 60 (20–106) | 43 (9–60) | 36 (20–60) | 80 |
| | 5 | 6 | 150 (90–293) | 44 (11–105) | 65 (40–95) | 130 |
| *p-cyclo*-Hexyloxy-α-phenyl-ethyl-amine HCl | 5 | 6 | 122 (78–172) | 0 | 5 | 15 |
| | 10 | 6 | 147 (95–236) | 45 (0–153) | 15 (8–25) | 40 |
| | 15 | 7 | 222 (120–331) | 60 (0–177) | 11 (7–20) | 50 |
| *m-cyclo*-Hexyloxy-α-phenyl-ethyl-amine HCl | 10 | 3 | 130 (103–164) | 85 (46–136) | 17 (15–20) | 40 |

* Range in parenthesis.

gesic activity of both *cyclo*hexyl ethers by about one hour.

*Tolerance to the Analgesic Response.*—Six rats were injected daily for four weeks (except Sundays) with the *p-cyclo*hexyl ether (15 mg./kg.). The analgesic response was measured weekly.

TABLE III

THE EFFECT OF CONTINUED DOSAGE (15 MG./KG. I.P.) ON THE ANALGESIC ACTIVITY OF *p-CYCLO*HEXYLOXY-α-PHENYLETHYLAMINE HCl IN RATS

Mean of 4 rats. Ranges are given in parentheses. The "normal" threshold is proportional to the heat delivered by the lamp.

| Time (weeks) | Wt. (g.) | "Normal" Threshold | Max. % Elevation of Reaction Threshold |
|---|---|---|---|
| 0 | 368 | 63 (58–72) | 296 (192–353) |
| 1 | 359 | 88 (69–132) | 179 (30–300) |
| 2 | 328 | 114 (72–144) | 164 (14–274) |
| 3 | 344 | 117 (100–144) | 60 (0–100) |
| 4 | 334 | 123 (79–156) | 61 (17–52) |

One rat died of infection and another was rejected because of sluggish reactions. The averaged data for the remainder are summarized in Table III. Complete tolerance to the drug was not established during four weeks, and the observed reduction in % elevation of reaction threshold is due in some measure to the progressive rise in "normal" threshold. There was little alteration in duration of effect or in the time taken to reach the peak response, but whereas the animals initially showed strong depression after the daily dose, two weeks later this was replaced by a short phase of excitability followed by mild depression. No abstinence syndrome was observed on cessation of dosage and the animals remained healthy for some weeks.

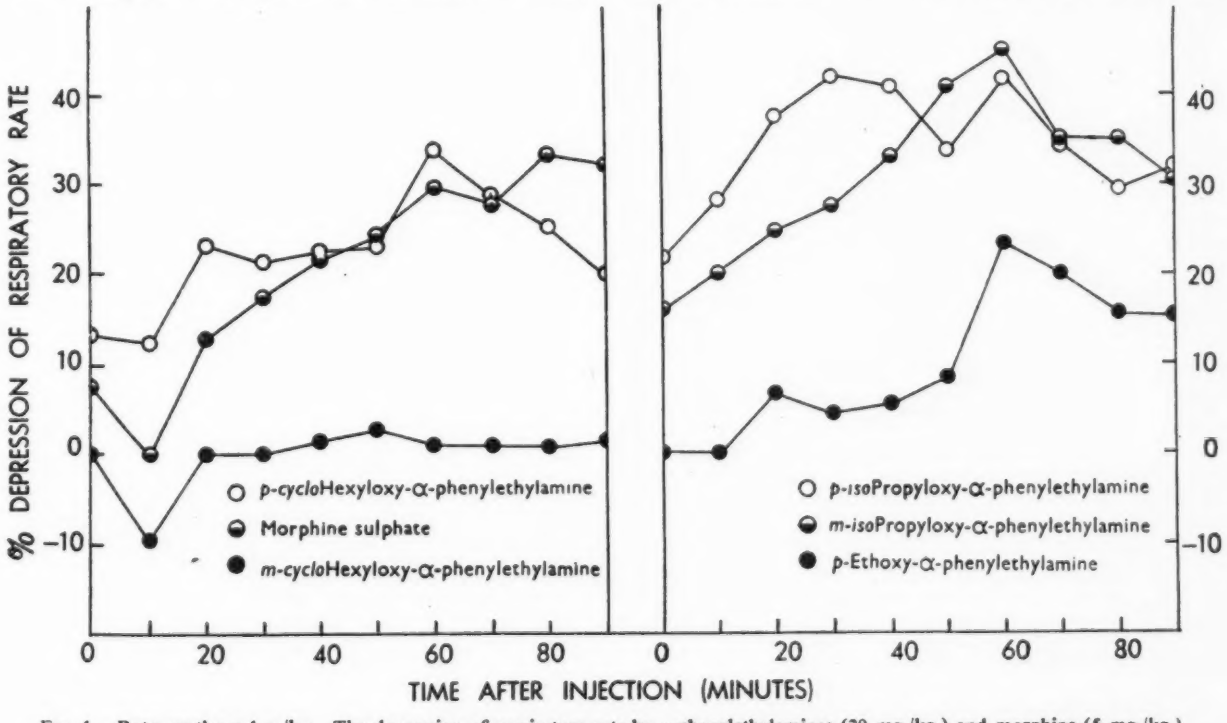


FIG. 1.—Rats, urethane 1 g./kg. The depression of respiratory rate by α-phenylethylamines (20 mg./kg.) and morphine (5 mg./kg.) after intraperitoneal injection. The ordinates represent the mean percentage increase in the time taken for 25 breaths in eight rats compared to a similar group receiving saline. The curves do not start at a common point owing to the large variation in respiratory rate in individual rats; the rate of the controls is taken as zero.

*Respiratory Depression.*—The rate of respiration has been measured by recording chest movements by a thread and lever writing on a kymograph drum. Urethane (1 g./kg. i.p.) was used to immobilize the animals. In eight controls there was no depression of rate for the first hour. Eight rats were used for each amine (except the *p*-ethoxy ether ; five) and the mean depressions of rate are plotted in Fig. 1. The *iso*propyl ethers were most active and gave strong depression lasting about 90 minutes.

*Blood Sugar.*—No significant effect could be recorded in groups of five rats after a dose of the amines (20 mg./kg.) or morphine (10 mg./kg.), determinations being made at half-hourly intervals, on blood drawn from the tail tip, by the method of Nelson (1944) and the single copper reagent of Somogyi (1945). Rabbits similarly gave no significant response. There was considerable difficulty in obtaining rat blood after the *p-cyclo*hexyl ether.

*Body Temperature.*—The rectal temperatures of groups of five rats were taken at intervals of 15 minutes after injection. A short training period was given on each of two days before the experiment. Saline gave small insignificant rises in temperature, whereas the amines (20 mg./kg.) and morphine (5 mg./kg.) consistently gave a fall 30 minutes after injection lasting two hours. Significant falls of 1–1.5° C. were obtained with both *cyclo*hexyl ethers ($P=0.01$) and with morphine ($P=0.05$). The significance of the falls due to the *iso*propyl and ethyl ethers was lower ($P=0.1$). The falls in temperature after the *cyclo*hexyl ethers and morphine were significant during the phase of maximal respiratory depression (which occurred much later than the analgesic maximum). There was no such correlation with the *iso*propyl ethers in spite of their powerful effect on respiration.

*Blood Pressure.*—The method of Crawford and Outschoorn (1951) for the rat was followed except that the vagi were not cut and the tail vein was found to be more convenient for injection. Similar results were obtained for each drug in two or more animals. Saline gave no response. The *cyclo*hexyl ethers (0.5–1 mg.), like morphine, produced an immediate fall of blood pressure of 10–20 mm. Hg, followed by a secondary rise. The fall in blood pressure was transitory and some degree of acute tolerance to subsequent doses was shown. Similar responses were obtained in the spinal cat. The *p-cyclo*hexyl ether produced a temporary apnoea at the same time as the rapid fall. The ethyl ether (1 mg.) produced an immediate transient rise in blood pressure of approximately 10 mm. Hg. The *iso*propyl ethers (0.5–1 mg.) had no immediate effect, but after some five minutes sudden rises in blood pressure, often paroxysmal, occurred, followed usually by death of the animal.

*Smooth Muscle.*—The spasm of rat duodenum in Tyrode (25 c.c.) due to acetylcholine (1 $\mu$g.) was not appreciably affected by the *iso*propyl and ethyl ethers. The *cyclo*hexyl ethers had slight activity at 400 $\mu$g., whereas pethidine completely relaxed the muscle at 100 $\mu$g.

## Discussion

Antipyretics give poor responses in analgesic assays using a heat stimulus (Smith, D'Amour, and D'Amour, 1943 ; Birren, Schapiro, and Miller, 1950), but the analgesic activity of aminopyrine by these methods is greater than that of its less basic analogue, phenazone. This suggested that the strongly basic nature of the alkoxy $\alpha$-phenylethylamines might account for their activity in assays where phenacetin gives poor results. However, the pharmacological similarity between morphine and *m*- and *p-cyclo*hexyloxy $\alpha$-phenylethylamine (Table IV), emphasized by the contrasting behaviour of the closely related *iso*propyl ethers, suggests that the mode of action of the *cyclo*hexyl ethers resembles that of morphine rather than the antipyretics. Similar qualitative concordance of properties has been described for pethidine (Yonkman, 1948) and amidone (Thorp, 1949 ; Scott and Chen, 1946 ; Chen, 1948), in spite of little discernible structural resemblance. The relatively inert nature of *p*-ethoxy $\alpha$-phenylethylamine, structurally analogous to phenacetin, stresses the dissimilarity to the antipyretics. In contrast to

Table IV

COMPARATIVE PHARMACOLOGY OF MORPHINE AND ALKOXY-$\alpha$-PHENYLETHYLAMINES

| Drug | Analgesic Activity (Heat Stimulus) | Nature of Convulsant Activity | Straub Sign (Mice) | Respiratory Depression | Blood Pressure | Body Temp. Reduction |
|---|---|---|---|---|---|---|
| Morphine | +++ | Tonic | + | ++ | Fall | + |
| *p-cyclo*-Hexyloxy- | ++ | Tonic | + | + | Fall | ++ |
| *m-cyclo*-Hexyloxy- | ++ | Tonic | + | − | Fall | + |
| *p-iso*-Propyloxy- | − | Clonic | ± | ++ | Rise | ± |
| *m-iso*-Propyloxy- | − | Clonic | − | ++ | Rise | ± |
| *p*-Ethoxy- | − | Clonic | − | + | Rise | ± |

Column 1 indicates the etherifying group of $\alpha$-phenylethylamine.

β-phenylethylamines, few α-phenylethylamines have been found to be analgesic. Like pethidine and amidone, *m-cyclo*hexyloxy α-phenylethylamine shows a formal resemblance to structures present in the morphine molecule.

Morphine has influence on the whole cerebrospinal axis. It might be expected that morphine or morphine-like drugs would show some difference in the time taken to reach peak responses for different effects which are mediated at different levels in this axis. In these experiments an attempt to standardize conditions with respect to the animal used, the dose employed, and route of administration was made to see if any such dissociation occurred. It was found that the peak analgesic response occurred appreciably before the peak of respiratory depression or reduction in body temperature. These two properties showed correlation in time in morphine and the *cyclo*hexyl ethers but not in the *iso*propyl ethers. It is generally assumed that the respiratory effects of morphine are mediated in the respiratory centres of the brain, whereas there is evidence that the responses used in heat stimulus assays are mediated by spinal reflexes. Spinal animals give the same type of response as intact animals (Bonnycastle and Leonard, 1950; Houde and Wikler, 1951; Irwin, Houde, Bennett, Hendershot, and Seevers, 1951). The observed dissociation in time fulfils expectation, but the reason can only form the subject of speculation.

Two generalizations have emerged from recent work on analgesics with which the results with the *cyclo*hexyl ethers agree. The assay of potency of analgesics by measurement of spinal reflex depression in animals, by dulling of experimental pain in man, and by relief of pain in clinical conditions give broadly parallel results. These methods probably involve distinct neuronal pathways. Secondly, analgesics show similar qualitative behaviour in properties unrelated to pain perception. These generalizations hold good although there is no real chemical relationship between the different classes of analgesic. It seems reasonable to assume either that analgesics act on the same type of structure common to various parts of the central nervous system—e.g., Wikler (1950) suggests internuncial neurones—or that they act on physiological mechanisms common to different structures in the central nervous system.


## Summary

1. *m-* and *p-cyclo*Hexyloxy-α-phenylethylamine show qualitative similarity to morphine in analgesic activity, respiratory depression, effect on body temperature and blood pressure. No consistent response was obtained for blood sugar changes.

2. *m-* and *p-iso*Propyloxy- and *p*-ethoxy-α-phenylethylamine have no analgesic activity and, apart from respiratory depression their pharmacological properties are different from those of the *cyclo*hexyl ethers and morphine.

3. Evidence was obtained to show that the analgesic effect of the *cyclo*hexyloxy-α-phenylethylamines is more nearly related to that of morphine, amidone, and pethidine than to the chemically analogous antipyretic and analgesic phenacetin.


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# THE ACTION OF DRUGS ON ISOLATED MAMMALIAN BRONCHIAL MUSCLE

BY

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Recently the pharmacological preparation consisting of a chain of tracheal or bronchial rings has been much used (Castillo and de Beer, 1947). Results based on relaxation of tone suggest that the relative potencies of bronchodilators in guinea-pig tracheal chains are of the same order as those found in human bronchial preparations (Hawkins and Schild, 1951). We have studied the response to histamine and acetylcholine of the circular muscle of similar preparations made from several mammals. The relative dilator activities of sympathomimetic amines and aminophylline on the contracted tracheal muscle have been compared with those found on contracted human bronchial preparations.

## METHODS

Tracheae of cats, dogs, rabbits, guinea-pigs, and rats were removed immediately after death. All tissues were carefully dissected and cut into rings which were tied together in chains with loops of cotton. In most experiments the cartilage in each ring was cut so that only the mucosa and smooth muscle band were left joining each ring together. The mucosa was then removed to allow of greater freedom of movement of the muscle. One to six rings (total length 2 to 3 cm.) were suspended in an isolated organ bath (volume 15 ml.) at 37° C. containing oxygenated Tyrode solution. Human bronchi (internal diameter 5–10 mm.) were secured from the post-mortem room as soon as possible after death; the delay was as long as 10 hours on one occasion, but the other specimens were obtained not more than 7 hours after death. The responses were recorded with a very light writing lever (magnification $\times 10$) on a slowly revolving kymograph. Histamine acid phosphate and acetylcholine chloride were left in contact with the preparation for one to three minutes. The preparation was washed twice after the addition of each drug and usually returned to baseline within five minutes. Antagonist or bronchodilator drugs were introduced into the bath usually 30 seconds before the histamine or acetylcholine doses. Most preparations responded well for at least 6 hours, and many were used after 24 hours' storage at 4° C.

The drugs used were synthetic *l*-adrenaline, *l*-noradrenaline *d*-bitartrate monohydrate, *dl-iso*propylnoradrenaline hydrochloride (isoprenaline), and *l*-ephedrine hydrochloride. All solutions were made up in 0.01 N-HCl, and dilutions were made with distilled water. Aminophylline (Burroughs Wellcome) was also studied. Drug concentrations are given in terms of the active base.

## RESULTS

### *Action of Acetylcholine*

Acetylcholine causes contraction of all the chains, and recovery to the baseline is rapid when the bath fluid is changed. Effective concentrations are: dog, $10^{-8}$; cat, $10^{-7}$; rabbit, $10^{-6}$; rat, guinea-pig, and human, $10^{-5}$.

For the comparative assay of bronchodilator drugs, the dose of adrenaline necessary to reduce by 50% the contraction produced by acetylcholine was taken as unity. Two dose levels of acetylcholine and antagonist were usually used.

The results for all the bronchodilators studied are shown in Table I. Each value is the geometric mean of at least six separate experiments. The results on the human bronchi showed considerable variation (see Table I for range), but this was much less in the animal experiments. Isoprenaline in every species tested was more active than adrenaline in reducing the acetylcholine-induced spasm (Fig. 1). Noradrenaline possesses about one-hundredth the activity of adrenaline on human tissue (Fig. 2), but it is equipotent in the rabbit and cat. The rabbit preparation is relatively insensitive to sympathomimetic amines, the adrenaline concentration for antagonism being as high as $10^{-6}$, whereas in the other preparations the corresponding concentration for antagonism is $10^{-8}$ to $10^{-7}$.

### *Action of Histamine*

When added to the bath, histamine causes contraction of preparations from some species. Recovery may be slow, however, when it is washed out of the human preparation (average concentra-

TABLE I

EQUIACTIVE DOSES OF BRONCHODILATOR DRUGS

Stimulants are histamine (Hist.) and acetylcholine (Ach.). Figures in brackets indicate range of individual results of human experiments.

| Preparation: | Human Bronchial Chain | | | Tracheal Rings | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Guinea-pig | | Dog | | Rat | Rabbit | Cat |
| Constrictor: | Hist. | Ach. | None* | Hist. | Ach. | Hist. | Ach. | Ach. | Ach. | Ach. |
| *dl*-Isoprenaline.. | 0.5 (0.25–1) | 0.4 (0.25–1) | 0.19 | 0.25 | 0.23 | 0.23 | 0.25 | 0.13 | 0.25 | 0.11 |
| *l*-Adrenaline .. | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *l*-Noradrenaline | 100 (50–200) | 75 (20–200) | 23(*dl*) | 5 | 5 | 2 | 2 | 2 | 1 | 1 |
| *l*-Ephedrine .. | 1,700 (500–2,000) | 1,200 (500–2,000) | 710 | 470 | 490 | 500 | 500 | 400 | 290 | 280 |
| Aminophylline.. | 500 (250–1,000) | 2,300 (1,250–7,500) | 1,900 | 1,800 | 10,300 | 1,600 | 10,100 | 7,300 | 10,000 | 15,400 |

* Hawkins and Schild (1951).

tion $10^{-5}$). Histamine ($10^{-6}$) stimulates the guinea-pig and dog preparations and the relative potencies of the bronchodilators (except aminophylline) are similar to those found for reducing the acetylcholine response (Table I). When a mixture of equal parts of acetylcholine and histamine is used as constrictor agent, similar results are also obtained. Most of the rat preparations are insensitive to histamine. Tracheal chains of the rabbit and cat are always insensitive to histamine, even in strong concentrations ($10^{-3}$).

*Action of Aminophylline*

The relative potency of aminophylline as a bronchodilator is also shown in Table I. Since the standard adrenaline dose in any one species is about the same for antagonism of the acetylcholine and the histamine responses, it will be seen that aminophylline is a stronger antagonist to histamine contraction than it is to that produced by acetylcholine.

FIG. 1.—Dog tracheal preparation in 15 ml. bath. Inhibition of acetylcholine contractions (0.1 μg.) by adrenaline (A, 0.4 μg.), noradrenaline (N, 0.8 μg.), isoprenaline (Iso, 0.1 μg.), and ephedrine (E, 100 and 200 μg.) Time of contact, 1 min.

## DISCUSSION

We have found that the human bronchial and the guinea-pig and dog tracheal preparations are usually sensitive to histamine, while those of the cat and rabbit entirely lack this property. There is no obvious explanation for this finding; it is not related to the histamine content of the tissues, which is relatively high in all species. The preparations reacting to histamine, however, are more sensitive to the sympathomimetic amines than are those of the cat and rabbit.

Isoprenaline is the most potent of all the bronchodilators studied, a result which agrees with the findings of previous workers using perfused guinea-pig lungs (Lands, Luduena, Ananenko, and Grant, 1950) or histamine aerosols in guinea-pigs (Chen, Portman, Russell, and Ensor, 1951). Hawkins and Schild (1951) suggest that the sympathomimetic amines and aminophylline exert a direct action on human bronchial muscle, so that isoprenaline is more active than adrenaline in this effect. Isoprenaline is much less active than adrenaline in producing relaxation of the fresh rabbit ileum (McDougal and West, 1952), so that it is possible that the point of action of these two amines is not the same in different types of smooth muscle.

The activity of noradrenaline relative to that of adrenaline varies with the species used, and it is far less in the human than in the other preparations. Hawkins and Schild (1951) found that the racemic form was about twenty-three times less active than adrenaline in producing relaxation of

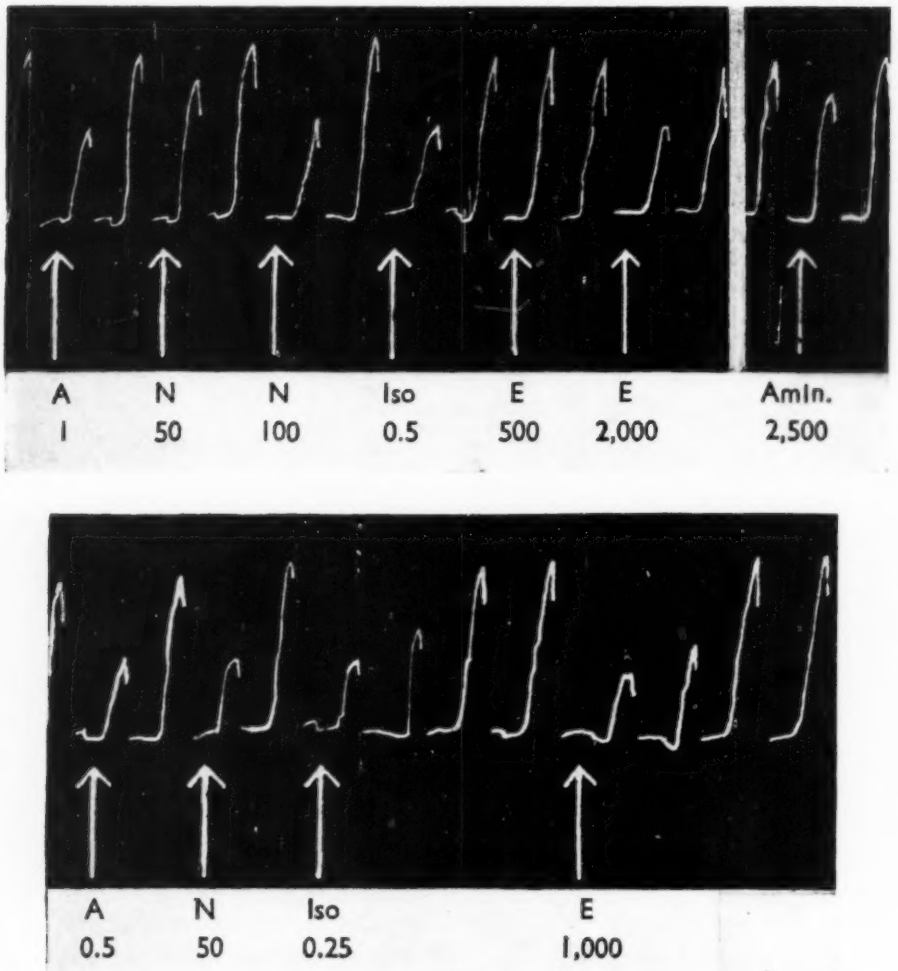


FIG. 2.—Human bronchial preparation in 15 ml. bath. Top tracing: inhibition of acetylcholine contractions (10 μg.) by adrenaline (A, 1 μg.), noradrenaline (N, 50 and 100 μg.), isoprenaline (Iso, 0.5 μg.), ephedrine (E, 500 and 2,000 μg.), and aminophylline (Amin., 2,500 μg). Lower tracing: inhibition of histamine contractions (20 μg.) by adrenaline, noradrenaline, isoprenaline, and ephedrine. Time of contact, 1 min.

the human bronchial chain. Our results with the laevo-form show it to be at least one hundred times less active, whether the constrictor be histamine or acetylcholine. Using an *in vivo* test (antagonism of the lethal effects of a histamine aerosol in guinea-pigs), Chen, Portman, Russell, and Ensor (1951) found that *l*-noradrenaline is sixty times less active than adrenaline, whereas earlier Tainter, Pedden, and James (1934) and Luduena (1942) had reported that *dl*-noradrenaline was only seven times less active in antagonizing the histamine constriction of perfused guinea-pig lungs. The relative activity of noradrenaline therefore varies not only with the species used but also with the preparation. In man, it is much weaker than adrenaline in its action on the bronchi, whereas in the rabbit and cat it is equally potent.

In agreement with the results of previous workers, we have found that ephedrine possesses a weak bronchodilator property, especially in the human preparations. It is interesting to note, however, that like the other sympathomimetic amines it is equally potent against the effects of histamine and acetylcholine. Aminophylline, on the other hand, is more effective in preventing a histamine than an acetylcholine bronchospasm.

Large doses of ephedrine antagonize the action of adrenaline on smooth muscle, probably by combining with the adrenaline receptors (for references, see Ambache, 1951). On the other hand, effective concentrations of isoprenaline do not influence the pressor response of adrenaline in dogs (Lands, Luduena, Ananenko, and Grant, 1950). In several preparations of the bronchial muscles of

each species, therefore, we have studied the effects of continuous infusions of ephedrine and of isoprenaline on the normal antagonism of all the amines to acetylcholine and histamine. The action of aminophylline was unaffected in all preparations, whereas the bronchodilator actions of adrenaline and noradrenaline were completely prevented ; the results with acute doses of ephedrine and isoprenaline were inconsistent. This evidence suggests that the site of action of aminophylline is different from that of the sympathomimetic amines.


## Summary

The relative bronchodilator actions of adrenaline, noradrenaline, isoprenaline, ephedrine, and aminophylline have been determined on the isolated tracheal rings of the guinea-pig, rat, cat, dog, and rabbit, and on the isolated human bronchial chain, after contraction of the tissues by histamine or acetylcholine. Whereas the guinea-pig, dog, and human preparations are sensitive to histamine, those of the cat and rabbit are insensitive.

Isoprenaline is the most active bronchodilator in the preparations whether the constrictor agent is acetylcholine or histamine. Although aminophylline usually is the weakest bronchodilator, it is unaffected by continuous infusions of effective concentrations of isoprenaline or ephedrine.

Noradrenaline is about one hundred times less active than adrenaline in preventing constriction of the human bronchial chain.


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# INVESTIGATION *IN VIVO* OF NEW INHIBITORS OF HYALURONIDASE

BY

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New polymeric condensation products containing diphenylmethane and triphenylmethane units and possessing the power to inhibit hyaluronidase *in vitro* were recently described by one of us (Hahn, 1952). The present report is concerned with the inhibitory effect of some substances of this type *in vivo* as well as their anti-inflammatory action, as measured by the reduction of oedema produced by injection of egg-white. The compounds were also studied for toxicity.

## MATERIALS AND METHODS

We selected for test four *triphenylmethane derivatives*, polycondensed trihydroxytricarboxytriphenylmethane ("Tri-*para*hydroxybenzoic acid," Compound 20 P), polycondensed hexahydroxytricarboxytriphenylmethane ("Trigentisic acid," Compound 21 P), and two polycondensed heptahydroxytricarboxytriphenylmethanes ("Di-β-resorcylic-gallic acid," Compound 16 P, and "Digentisic-gallic acid," Compound 30), as well as two *diphenylmethane derivatives*, polycondensed dihydroxydicarboxydiphenylmethane ("Di-*para*hydroxybenzoic acid," Compound 2 P) and polycondensed tetrahydroxydicarboxydiphenylmethane ("Digentisic acid," Compound 7 P). For purposes of comparison two simple hydroxybenzoic acids, *p*-hydroxybenzoic acid and gentisic acid, were also tested.

The diphenylmethane derivatives were prepared by condensing hydroxybenzoic acids with formaldehyde under conditions favouring the formation of products of fairly high molecular weight. The triphenylmethane derivatives were obtained by substitution in the methylene groups of polycondensed diphenylmethane derivatives by hydroxycarboxyphenyl radicals.

*Measurement of Inhibitory Power* in Vitro.—The *in vitro* activity was determined viscosimetrically with resorcinol as standard. A solution of testes hyaluronidase, extracted and purified by the method of Hahn (1943), was mixed with a neutral solution of the substance to be tested. After 30 minutes' incubation the mixture was added to a 0.2% solution of hyaluronic acid from umbilical cord, which was adjusted to *p*H 7 by means of phosphate. Readings were taken at 37° C. after different intervals. That concentration of the compound that was necesssary to reduce the rate of hydrolysis of hyaluronic acid by hyaluronidase to one-fifth was determined. This concentration was found to be 0.2% for resorcinol. A corresponding effect can be obtained with a 1% solution of sodium salicylate.

*Measurement of Inhibitory Power* in Vivo.—The inhibitory effect was estimated by a method based on the action of hyaluronidase on the rate of absorption of drugs injected subcutaneously. It permitted quantitative determination of the minimum dose capable of producing practically complete inhibition.

Female mice weighing 20–22 g. were used. The animals were of the same breed, they had been brought up on a uniform diet under identical conditions, and the environmental temperature was maintained at 21–22° C. All experiments were performed on groups of 5 mice between noon and 6 p.m. Urethane in a dose of 1.2 mg./g. body weight was injected subcutaneously as a 10% aqueous solution, and the interval was noted between the injection and the production of anaesthesia, which was said to be complete when the animal offered no resistance to being placed on its side. This interval will be referred to as the "absorption time of urethane." (It also includes the time necessary for urethane to exert its effect on the brain cells.)

A dose of 0.01 ml./g. body weight of a hyaluronidase solution containing 380 TRU/ml. was injected into one of the tail veins, the intravenous route being chosen in order to obtain a generalized effect of the hyaluronidase. Two minutes later the animals received urethane subcutaneously. The dose of hyaluronidase given was sufficient markedly to decrease the absorption time of urethane.

The inhibitory activity of the substances tested was assessed by their power to counteract the increase in the rate of absorption of urethane produced by hyaluronidase. They were dissolved in a dilute solution of NaOH and the *p*H was adjusted to 7. Forty to fifty minutes before the injection of the hyaluronidase a varying dose of these solutions was administered subcutaneously to some mice and by mouth to others.

The minimum dose necessary to inhibit completely the reduction by hyaluronidase of the absorption time of urethane is expressed as M.I.D.$_{100}$. Every dose level used was tried on two groups of mice. As the M.I.D.$_{100}$ was approached the number of groups was increased to four. Control tests using either urethane only or hyaluronidase and urethane were performed simultaneously.

The activity of the inhibitors was also checked by the skin-spreading method on rabbits (Duran-Reynals, 1942).

*Anti-inflammatory Effect.*—This was assessed by the power to depress artificial oedema produced by the injection of egg-white into female rats weighing 60–90 g. (Selye, 1937; Halpern, 1949; Gross, 1950; Wilhelmi and Domenjoz, 1950). Fresh egg-white (0.1 ml.) was injected subcutaneously into the dorsal side of one hind paw and an equal quantity of saline into the other. Ninety minutes later the animals were killed with ether. Both hind legs were removed at the knee joint and weighed. The difference in weight was taken as a measure of the oedema produced by the egg-white.

An arbitrary dose of a solution of the inhibitor to be tested was injected subcutaneously, and again 30 minutes later. Immediately after the second injection 0.1 ml. egg-white was injected into the hind paw. The smallest dose of the inhibitor needed to diminish the oedema produced by egg-white was determined by trial and error. As the inflammatory action of egg-white varies from one egg to another, simultaneous control tests were always made with the white of the same egg. Every dose was tried in two or three groups of 5 rats each.

*Toxicity.*—The substance to be tested was emulsified in an aqueous solution of gum arabic and sugar. The concentration of the emulsion varied from 2 to 30% according to the dose used. Single doses of 0.01–0.05 ml./g. body weight of the emulsion were given by stomach tube to female mice weighing 20–24 g. As a rule six dosage levels were studied, each on six mice. The animals were then carefully observed for one week. When deaths occurred, they usually did so within 48 hours. The LD50 values were determined by the method of Behrens-Kärber.

In chronic toxicity studies the substances were given by mouth to groups of 10 animals. Female mice weighing 20–24 g., rats weighing 80–140 g., guinea-pigs weighing 250–300 g., and rabbits weighing 1.8–2.5 kg. were used. The substances were administered daily for 30 days. The volume of the daily dose of the emulsion was kept within 0.01–0.02 ml./g. body weight.

The substances were given to man in the form of tablets. Groups of 5 apparently healthy volunteers received two daily doses of 0.5 g. the first week, four daily doses of 0.5 g. the second week, four daily doses of 1 g. the third week, four daily doses of 1.5 g. the fourth week, and four daily doses of 2 g. the fifth week.

TABLE I

INHIBITION OF HYALURONIDASE AND TOXICITY

| Substances | Inhibitory Power *in vitro* in Relative Units (Resorcinol =1) | Hyaluronidase Inhibition *in vivo* M.I.D.$_{100}$ | | Acute Toxicity LD50 Per os μg./g. |
|---|---|---|---|---|
| | | s.c. μg./g. | Per os μg./g. | |
| No. 21 P "Trigentisic acid" | 2,485 | 10 | 50 | >20.000 |
| No. 20 P "Tri-*para*hydroxybenzoic acid" .. .. | 1,275 | 25 | 75 | 2.700 |
| No. 30 "Digentisic-gallic acid" .. .. | 2,275 | 25 | 75 | 12.500 |
| No. 16 P "Di-β-resorcylic-gallic acid" .. .. | 1,300 | 50 | 200 | 5.400 |
| No. 7 P "Digentisic acid" | 2,000 | 50 | 200 | 10.000 |
| No. 2 P "Di-*para*hydroxybenzoic acid" .. .. | 400 | 150 | 750 | 4.500 |
| Gentisic acid .. .. | 0.8 | —* | —* | 4.500 |
| *p*-Hydroxybenzoic acid .. | 0.1 | —* | —* | 4.800 |

* No effect in tolerated doses.

## RESULTS

The *in vitro* activity† of the compounds investigated is given in Table I.

### *Inhibition of the Increase by Hyaluronidase of the Rate of Absorption of Urethane*

In control experiments on 265 mice the absorption time of urethane administered subcutaneously was found to be $20.4 \pm 4.17$ minutes. The corresponding values for 240 mice pretreated with hyaluronidase were $8.7 \pm 1.64$. The hyaluronidase thus decreased the absorption time by about half. This difference was highly significant ($P<0.01$).

All the polycondensed substances tested reduced the action of hyaluronidase (Table I). Inhibition was said to be complete when the absorption time for the hyaluronidase treated animals exceeded 16 minutes. The triphenylmethane derivative, "Trigentisic acid," showed greatest activity. A dose of 10 μg./g. injected subcutaneously or 50 μg./g. administered by mouth was sufficient to abolish the effect of hyaluronidase injected intravenously. Of the two diphenylmethane derivatives the more active was "Digentisic acid." The inhibitory power of "Di-*para*hydroxybenzoic acid" was much weaker and only slightly stronger than that of heparin. The inhibitory action of heparin on hyaluronidase has been described by earlier workers (McClean, 1942; Rogers, 1946) and was included here only for purposes of comparison. The M.I.D.$_{100}$ of heparin, when administered subcutaneously, was found to be 200 μg./g. Tolerable doses (max. 1,000 μg./g.) of gentisic acid and *p*-hydroxybenzoic acid produced no signs of inhibition.

† The *in vitro* experiments were performed in co-operation with J. Fekete and E. Frank. A detailed report is in preparation.

The dose-response curves for the various inhibitors are given in Fig. 1, from which it is clear that the effect increased with increasing dosage only up to the level of the M.I.D.$_{100}$. Every substance was also tested in animals not treated with hyaluronidase. In these control experiments we used a dose of the inhibitors five times as large as the M.I.D.$_{100}$. The purpose of these experiments was to determine whether the substances influence the rate of absorption of urethane and whether they exert any analeptic or anaesthetic action. None of the compounds tested showed any such effects.

*Inhibition of the Skin-spreading Effect of Hyaluronidase*

Although the skin-spreading technique is less suitable for quantitative determinations, the inhibitory effect of these new polycondensed products was checked by the skin-spreading technique, and it was found that an oral dose of 500 μg./g. of any of them was sufficient markedly to inhibit the skin-spreading effect of hyaluronidase.

*Anti-inflammatory Action*

Various doses of the following substances were tested for their anti-inflammatory action, as measured by the reduction of oedema produced by the injection of egg-white into rats: "Trigentisic acid," "Tri-*para*hydroxybenzoic acid," "Digentisic acid," "Di-*para*hydroxybenzoic acid," gentisic acid, and *p*-hydroxybenzoic acid. The results are given in Table II.

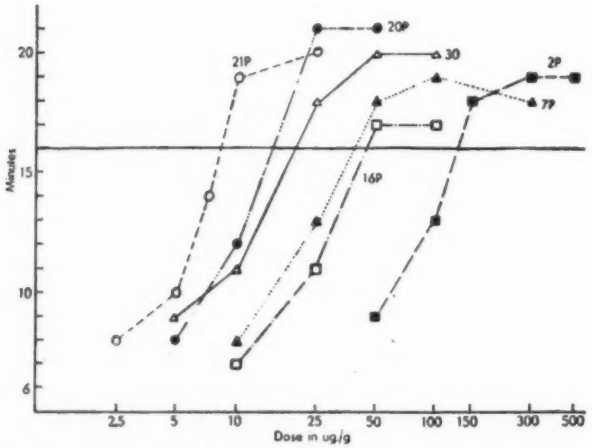


FIG. 1.—Dose-response curves for polycondensed diphenylmethane and triphenylmethane derivatives injected subcutaneously. Ordinate: rate of absorption of urethane (1.2 mg./g. s.c.) given two minutes after hyaluronidase (3.8 TRU/g. i.v.), as judged by the onset of anaesthesia. Abscissa: dose of inhibitor (μg./g.). No. 21 P "Trigentisic acid." No. 20 P "Tri-*para*hydroxybenzoic acid." No. 30 "Digentisic-gallic acid." No. 16 P "Di-β-resorcylic-gallic acid." No. 7 P "Digentisic acid." No. 2 P "Di-*para*hydroxybenzoic acid."

TABLE II

EFFECT ON THE OEDEMA PRODUCED BY INJECTION OF EGG-WHITE INTO THE HIND PAWS OF RATS
Each inhibitor was given subcutaneously in two doses, the first dose half an hour before, and the second dose simultaneously with, the injection of 0.1 ml. egg-white. Neither gentisic acid nor *p*-hydroxybenzoic acid had any action in these doses.

| Dose s.c. μg./g. | % Depression of Oedema | | | |
|---|---|---|---|---|
| | No. 21 P "Trigentisic Acid" | No. 20 P "Tri-*para*hydroxybenzoic Acid" | No. 7 P "Digentisic Acid" | No. 2 P "Di-*para*hydroxybenzoic Acid" |
| 2×5 | 0 | 0 | 0 | 0 |
| 2×12.5 | 25 | 0 | 0 | 0 |
| 2×25 | 32 | 16 | 14 | 0 |
| 2×50 | 36 | 30 | 29 | 0 |
| 2×100 | 35 | 36 | 35 | 33 |
| 2×250 | 41 | 32 | 33 | 35 |
| 2×500 | 37 | 40 | 38 | 33 |

Doses of about the same size as those which just inhibited hyaluronidase *in vivo* reduced the oedema by 25–45%. Larger doses produced no further reduction. The minimum effective dose of "Trigentisic acid," was 2×12.5 μg./g.; the corresponding doses for "Tri-*para*hydroxybenzoic acid" and for "Digentisic acid" were 2×50 μg./g. and for "Di-*para*hydroxybenzoic acid" 2×100 μg./g. "Di-β-resorcylic-gallic acid" and "Digentisic-gallic acid" were only tried in doses of 2×250 μg./g. and more, and produced a pronounced anti-inflammatory effect. Gentisic acid and *p*-hydroxybenzoic acid had no obvious effect on the inflammatory reaction, even in doses of 2×500 μg./g.

*Toxicity*

The acute oral toxicity values are given in Table I. "Trigentisic acid" was least toxic. Daily doses of 200 μg./g. for 30 days of "Trigentisic acid," "Digentisic-gallic acid," and "Digentisic acid" produced no manifest signs of intoxication. "Trigentisic acid" was also administered daily in a dose of 400 μg./g. for 30 days without toxic effects. A daily dose of 200 μg./g. "Di-β-resorcylic-gallic acid," "Tri-*para*hydroxybenzoic acid," and "Di-*para*hydroxybenzoic acid" caused diarrhoea and moderate loss of weight.

In man "Trigentisic acid" and "Digentisic acid" were given daily in doses increasing up to 8 g. and were well tolerated. In corresponding trials with "Di-β-resorcylic-gallic acid" a daily dose of 6 g. caused nausea and diarrhoea in some volunteers. These side-effects were also produced by a daily dose of 4 g. "Di-*para*hydroxybenzoic acid."

## DISCUSSION

All the polycondensed diphenylmethane and triphenylmethane derivatives possessing the power to inhibit hyaluronidase *in vitro* and studied in the

present investigation were found to be highly inhibitory *in vivo*, too. On the other hand, gentisic acid and *p*-hydroxybenzoic acid, which were only one-thousandth to one-hundredth as active *in vitro* as the polycondensed products, appeared to exert no inhibitory effect *in vivo*. A question that naturally presents itself is whether the inhibitory activity of the polycondensed compounds *in vivo* bears any quantitative relationship to their activity *in vitro*.

" Trigentisic acid " showed greatest activity both *in vitro* (2,485 rel. units) and *in vivo* ($M.I.D._{100}$, 10 $\mu$g./g. s.c.). " Di-*para*hydroxybenzoic acid " showed least activity *in vitro* (400 rel. units) and *in vivo* ($M.I.D._{100}$, 150 $\mu$g./g. s.c.). The *in vitro* activity of the remaining compounds varied between 1,275 and 2,275 relative units and the $M.I.D._{100}$ *in vivo* between 25 and 50 $\mu$g./g. s.c. Within these limits no quantitative relationships were observed between the *in vitro* and the *in vivo* activities of the compounds. In evaluating these observations the possible influence of variation in the rate of absorption, breakdown, and excretion, and other factors liable to blur the picture, must be borne in mind.

As to the anti-inflammatory effect it should be stressed that the polycondensed compounds tested reduced artificial oedema in rats and that the smallest dose capable of producing maximum reduction of the oedema was approximately equal to that required completely to abolish hyaluronidase activity *in vivo*. This minimum dose reduced the inflammatory reaction by 30 to 40%, and a ten- to twenty-fold increase of the dose caused no further depression. These observations suggest that the anti-inflammatory action of the substances is related to their inhibitory effect on hyaluronidase and that hyaluronidase activity is involved in the mechanism of inflammation, at least of the type described here.

Since these compounds can affect hyaluronidase activity and inflammation *in vivo*, it may be that they can exert the same effect in those pathological conditions in which hyaluronidase is assumed to be involved. So far nothing is known of the action of inhibitors on the hyaluronidase normally present in the body. The fact that the synthetic inhibitors did not retard the absorption of urethane in normal animals that had not received hyaluronidase does not necessarily mean that they do not inhibit the hyaluronidase normally present in the body ; it is possible that body hyaluronidase plays so small a rôle in absorption that any elimination of its effect does not produce demonstrable changes in the rate of absorption of urethane, for instance. It is also possible that normally the body hyaluronidase is practically completely inhibited, or that the subcutis, capillaries, and the blood-brain barrier contain no hyaluronidase.

As some of the substances described possessed low toxicity and a marked power to inhibit hyaluronidase *in vivo*, with an anti-inflammatory effect, they may open a new approach to the therapy of pathological conditions in which the hyaluronic acid-hyaluronidase system is involved. From a therapeutic point of view " Trigentisic acid " seems to be the most promising compound. It showed not only greatest inhibitory power *in vitro* and *in vivo*, but also the lowest toxicity in animals and in man. In animal experiments doses 400 times larger than the $M.I.D._{100}$ were well tolerated.

## Summary

1. Six new polycondensed diphenylmethane and triphenylmethane derivatives possessing a strong inhibitory effect *in vitro* on hyaluronidase were tested for their inhibitory effect *in vivo*, for their anti-inflammatory action, and for their toxicity.

2. All of the substances were found to possess marked *in vivo* activity as judged by their inhibition of the effect of hyaluronidase on absorption of urethane injected subcutaneously into mice. The most active substance, Compound 21 P (" Trigentisic acid "), practically completely inhibited hyaluronidase *in vivo* in a subcutaneous dose of 10 $\mu$g./g. and in an oral dose of 50 $\mu$g./g. body weight.

3. All the six polycondensed substances reduced the artificial oedema caused by the injection of egg-white in rats. A certain parallelism was found between the intensity of this anti-inflammatory action and the inhibition of hyaluronidase.

4. Toxicity tests showed Compound 21 P (" Trigentisic acid ") to be least toxic to animals. LD50 by mouth in mice was more than 20 mg./g. Daily oral doses of 0.4 mg./g. for 30 days produced no signs of intoxication in mice, rats, guinea-pigs, and rabbits. In human beings a daily oral dose of 8 g. produced no side-effects.

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# ANTIBACTERIAL ACTIVITIES OF SOME BIS*ISO*QUINOLINIUM SALTS

BY

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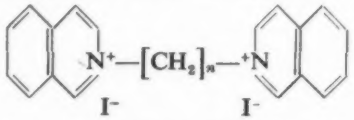
Buttle (private communication) observed that decamethylene bis*iso*quinolinium bromide (Compound No. 7, Collier and Taylor, 1949), which had been prepared in a research programme on neuromuscular blocking agents, possessed considerable antibacterial activity. It was therefore interesting to inquire how far the antibacterial activities of bis*iso*quinolinium salts were influenced by alterations in chemical structure, particularly by those factors that had been found to affect neuromuscular blocking activity. The effect of reduction of the heterocyclic nucleus was examined by comparing Compounds 7 and 8 and that of introduction of methoxyl groups by comparing Compounds 8, 15, and 20 (Taylor and Collier, 1950, 1951). In order to examine the effect of variations in length of the polymethylene chain we prepared a series of polymethylene bis*iso*quinolinium salts of general formula (I), where $n=8$–14,

(I) 


16, 18, or 20. Compound 7, rather than Compounds 8, 15, or 20, was chosen as the basis for this series, since it was the least toxic and was derived from most readily accessible starting material.

## METHODS

*In vitro* observations on the inhibitory activities of the *iso*quinolinium compounds and of cetyltrimethylammonium bromide (CETAB) were made with the following strains of bacteria: *Streptococcus pyogenes* (NCTC 8195); *Str. faecalis* (M75); *Staphylococcus aureus* (Oxford H); *Vibrio cholerae* (48210); *Salmonella typhi* (NCTC 5758); *Shigella shigae* (NCTC 4837); *Sh. flexneri* (NCTC 4832); *Pseudomonas pyocyanea* (NCTC 8099); and *Mycobacterium phlei* (NCTC 525). The strain of *Bacterium coli* used was one isolated at Ware from human faeces in 1947.

Drugs were serially diluted by 1 in 2 in 1% peptone (Difco)-water containing 0.5% dextrose and adjusted to *p*H 7.2. After autoclaving, the dilutions were inoculated with a washed suspension of a 22-hour culture of the bacterial species concerned and adjusted to give approximately 1,000 organisms per culture tube (5 ml.). Cultures were incubated at 37° C. and growth read by eye at 24 hours and subsequently. Results were expressed as the minimal inhibitory concentration (M.I.C.), which was the least concentration in which no growth was visible at 24 hours or, with *Myco. phlei*, at 5 days.

## RESULTS

### *Effect of Variation of End-group on Activity*

The *in vitro* activities of four decamethylene compounds and of CETAB against a variety of bacteria are expressed in Table I. Whereas the bis*iso*quinolinium compounds possess appreciable activity in all species except *Ps. pyocyanea*, none is as active as CETAB. It is difficult to draw systematic conclusions from these results, but two trends are apparent: (*a*) In two species of *Shigella* the methoxylated compounds (15 and 20) were appreciably less active than those without such groups (7 and 8); (*b*) in *Str. pyogenes* and in *Myco. phlei* the three bistetrahydro*iso*quinolinium compounds (8, 15, and 20) were more active than the bis*iso*quinolinium compound (7).

### *Effect of Chain-length on Activity*

The relationship of chain-length to activity is indicated in Fig. 1. In each bacterial species examined, increase in chain-length up to the octadecamethylene compound was accompanied by a steady enhancement of antibacterial activity. The eicosane compound exhibited no more and sometimes less activity than the octadecamethylene. A very similar trend against streptococci, staphylococci, and *B. coli* was found in the methonium series by Paton and Zaimis (1949).

The question arose as to whether the higher members of the bis*iso*quinolinium series should be investigated for *in vivo* antibacterial activity. Preliminary toxicity tests in mice of each member

TABLE I

ANTIBACTERIAL ACTIVITIES OF DECAMETHYLENE BIS*ISO*QUINOLINIUM DERIVATIVES
$\{R^+—[CH_2]_{10}—R^+\}2X^-$

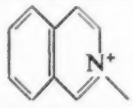
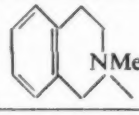
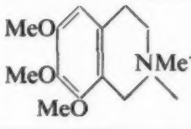
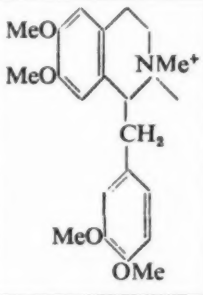
| Ser. No. | Structure of End-group R | M.I.C in μg. per ml. against | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Str. pyogenes* | *Str. faecalis* | *Staph. aureus* | *V. cholerae* | *S. typhi* | *Sh. shigae* | *Sh. flexneri* | *B. coli* | *Ps. pyocyanea* | *Myco. phlei* |
| 7 |  | 125 | 160 | 1.26 | 160 | 40 | 10 | 20 | 320 | >200 | >200 |
| 8 |  | 25 | 80 | 2.5 | 320 | 40 | 40 | 80 | 160 | >200 | 12.5 |
| 15 |  | 12.5 | 125 | 0.78 | >1,000 | 250 | 500 | 500 | 1,000 | >200 | 3.55 |
| 20 |  | 25 | 50 | 1.56 | 625 | 312 | 200 | 312 | >200 | >200 | 12.5 |
| | CETAB | 0.63 | 1.12 | 0.195 | 25 | 25 | 3.125 | — | 100 | >200 | 1.56 |

TABLE II

POLYMETHYLENE BIS*ISO*QUINOLINIUM IODIDES

| Value of *n* | Reaction Time (hr.) | Reaction Solvent | M.p. (d= decomp.) | Cryst. Form. | Crystn. Solvent | Found, %* | | | | Formula | Required, % | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | C | H | N | I | | C | H | N | I |
| 8 | 30 | Benzene | 258–259° (d) | Yellow rosettes or clusters | MeOH | 50.0 | 5.05 | 4.4 | 40.6 | $C_{26}H_{40}N_2I_2$ | 50.0 | 4.85 | 4.5 | 40.7 |
| 9 | 72 | ,, | 211–212° | Yellow needles | EtOH | 50.9 | 5.1 | 4.4 | 39.7 | $C_{27}H_{42}N_2I_2$ | 50.8 | 5.1 | 4.4 | 39.8 |
| 11 | 80 | ,, | 183–184° | Yellow granules | EtOH | 52.6 | 5.4 | 4.0 | 37.9 | $C_{29}H_{46}N_2I_2$ | 52.25 | 5.45 | 4.2 | 38.1 |
| 12 | 30 | ,, | 206–207° (d) | Lemon yellow microcrystals | MeOH-EtOH | 53.3 | 5.6 | 3.95 | 36.9 | $C_{30}H_{48}N_2I_2$ | 52.9 | 5.6 | 4.1 | 37.35 |
| 13 | 120 | ,, | 185–187° | Yellow granules | EtOH | 53.8 | 5.95 | 3.9 | 36.2 | $C_{31}H_{50}N_2I_2$ | 53.6 | 5.8 | 4.0 | 36.6 |
| 14 | 48 | ,, | 170–171° | ,, ,, | EtOH | 54.3 | 6.0 | 3.7 | 35.65 | $C_{32}H_{52}N_2I_2$ | 54.2 | 6.0 | 3.95 | 35.9 |
| 16 | 48 | Alcohol | 145.5–146.5° | Yellow nodules or rosettes of tiny needles | EtOH-$Et_2O$ | 55.2 | 6.3 | 3.7 | 34.2 | $C_{34}H_{56}N_2I_2$ | 55.4 | 6.3 | 3.8 | 34.5 |
| 18 | 72 | ,, | 138–140°† | Yellow powder‡ | EtOH-$Et_2O$ | 56.8 | 6.35 | 3.6 | 33.0 | $C_{36}H_{60}N_2I_2$ | 56.5 | 6.6 | 3.7 | 33.25 |
| 20 | 120 | ,, | 143.5–144.5° | Yellow needles§ | EtOH-$Et_2O$ | 57.4 | 7.2 | 3.7 | 31.45 | $C_{38}H_{64}N_2I_2$ | 57.6 | 6.9 | 3.5 | 32.1 |

* Dried at 100° *in vacuo*. †After drying at 100° *in vacuo*. The air-dried salt sinters at *ca.* 75–77°, hardens and again softens at *ca.* 116–118°, again hardens and melts at 138–140°. ‡ Contains solvent of crystallization—lost 3.65% by weight on drying *in vacuo* at 100°. § Contains solvent of crystallization—lost 6.0% by weight on drying *in vacuo* at 100°.

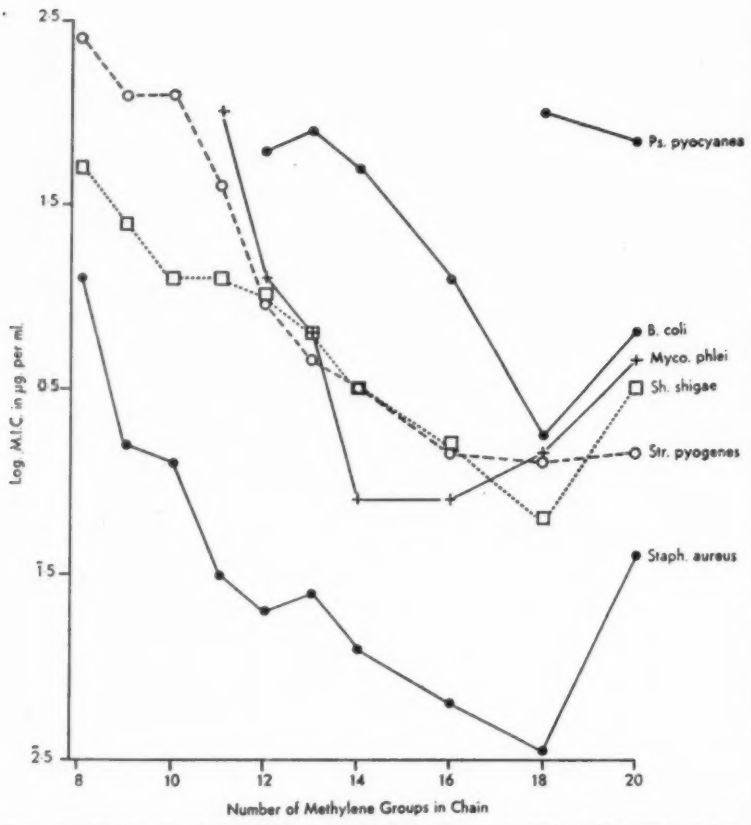



FIG. 1.—Effect of chain-length on activities of polymethylene bis*iso*quinolinium compounds against six species of bacteria. Log M.I.C. (µg. per ml.) plotted against the number of methylene groups in the polymethylene chain.

of the series, however, showed that all compounds possessed considerable toxicity and that there was a steady increase in toxicity with increase in chain-length. For example, while mice tolerated subcutaneous doses of 40 mg. per kg. of the octa- and nona-methylene compounds, they tolerated 5 but not 10 mg. of the octadecamethylene and eicosane compounds. The high toxicity of these compounds, increasing with increased antibacterial activity, suggested that *in vivo* antibacterial tests would be fruitless.

## CHEMICAL SECTION

(Microanalyses are by Drs. Weiler and Strauss, Oxford. All melting points are uncorrected.)

The polymethylene glycols required in this work have all been described in the literature (Chuit, 1926; Chuit and Hausser, 1929; Ashton and Smith, 1934; Shiina, 1937; Bouveault and Blanc, 1903). The m.p. of tetradecamethylene glycol was found to be 86.5–87° C., whereas Chuit (1926) recorded an m.p. of 85° C. (Found: C, 73.0; H, 13.1. Calc. for $C_{14}H_{30}O_2$: C, 73.0; H, 13.15%). An apparently unrecorded intermediate in the synthesis of eicosane diol is 1:18-*dicyano-octadecane*, which crystallized from light petroleum (b.p. 40–60° C.) in long flat needles, m.p. 66–67° C. (Found: C, 78.9; H, 11.7; N, 9.3. $C_{20}H_{36}N_2$ requires C, 78.9; H, 11.9; N, 9.2%). Treatment of the glycols with excess hydriodic acid (sp. gr., 1.94) yielded the corresponding polymethylene di-iodides, all of which were known (Ashton and Smith, 1934; Shiina, 1937; v. Braun and Danziger, 1912; v. Braun, 1909; Ziegler and Weber, 1937) with the exception of the following, to which no references could be found in the literature: *tridecamethylene di-iodide*, small needles, m.p. 39–40° C., from alcohol (Found: C, 35.9; H, 6.2; I, 57.7. $C_{13}H_{26}I_2$ requires C, 35.8; H, 6.0; I, 58.3%), and *tetradecamethylene di-iodide*, plates, m.p. 49–50° C., from alcohol (Found: C, 37.4; H, 6.1; I, 56.2. $C_{14}H_{28}I_2$ requires C, 37.3; H, 6.3; I, 56.4%). *Hexadecamethylene di-iodide*, plates, m.p. 55–56° C., from alcohol (Found: C, 40.5; H, 6.8; I, 52.9. $C_{16}H_{32}I_2$ requires C, 40.2; H, 6.7; I, 53.1%), is mentioned by Ziegler and Weber (1937), but no physical properties are given.

The *polymethylene bisisoquinolinium salts* were prepared by boiling the appropriate polymethylene di-iodide with 50% excess of *iso*quinoline in either benzene or alcohol solution for from 30–120 hours; the reaction mixture was cooled and filtered, and the product recrystallized. With the hexadecamethylene and octadecamethylene derivatives, it was necessary to add ether to the alcoholic reaction mixture in order to precipitate the crude quaternary salt.

The following decamethylene derivatives have already been described in connection with other work (Taylor, 1951, 1952): the bis*iso*quinolinium dibromide (Cpd. 7), the bis-(1:2:3:4-tetrahydro-2-methyl*iso*quinolinium) di-iodide (Cpd. 8), the bis (1:2:3:4-tetrahydro-6:7:8-trimethoxy-2-methyl*iso*quinolinium) di-iodide (Cpd. 15), and the bis [1-(3′:4′-dimethoxybenzyl)-1:2:3:4-tetrahydro-6:7-dimethoxy-2-methyl*iso*quinolinium] dimethosulphate (Cpd. 20). The properties of the other salts are given in Table II.

## SUMMARY

1. A series of polymethylene bis*iso*quinolinium salts, with 8–14, 16, 18, and 20 methylene groups, has been prepared. These were examined *in vitro* for inhibitory activity against 6 species of bacteria.

2. In all species inhibitory activity increased with increase in chain-length up to the octadecamethylene compound. The toxicity to mice showed a similar trend.

3. The inhibitory activities of four decamethylene bis*iso*quinolinium compounds that had previously been prepared were examined *in vitro* in ten species of bacteria. In two species of *Shigella* the methoxylated compounds were appreciably less active than those without such groups. In *Str. pyogenes* and *Myco. phlei* the three tetrahydro compounds were more active than the unreduced compound.

4. The antibacterial activities of the compounds examined do not appear sufficient, in view of their relatively high toxicities, to encourage prospects of their being useful against bacterial infections in man. The antibacterial activity of Compound 20, however, may be of some practical importance in maintaining sterile solutions of this drug, which has been found an effective muscle-relaxant in man (Bodman, Morton, and Wylie, 1952).

We are indebted to Mr. W. C. Austin and to Misses M. Boothroyd and E. M. Messham for technical assistance, and to the Directors of Messrs. Allen & Hanburys Ltd. for permission to publish this work.

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# THE EFFECT OF ADRENALINE AND NORADRENALINE ON THE ACTIVITY OF ISOLATED PREPARATIONS OF THE GUT FROM THE FOETAL GUINEA-PIG

BY

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A motor response to adrenaline which occurs in the terminal part of the guinea-pig ileum and is stronger in small than in large animals has previously been described (Munro, 1951a). In view of this apparent relation between the adrenaline response and the size of the animal, it was considered worth while making some observations on the response of the foetal gut to adrenaline. The first few observations suggested that the terminal part of the isolated foetal ileum was relaxed by adrenaline. Further work, however, showed that considerable differences in the nature and the degree of response to adrenaline occurred in different terminal segments. The response was often purely motor, as in the adult; but in those segments whose response was a relaxation continued washing, associated with a reduction of the prevailing tone, usually changed it to a contraction.

The present paper describes the distribution of the motor response to adrenaline in the foetal gut, and compares the characteristics of the intestinal response to adrenaline and noradrenaline. It has been shown by West and Shepherd (1951) that the foetal suprarenal glands contain a higher concentration of noradrenaline than of adrenaline. It was therefore of interest to determine the relative effects of these two substances on the foetal intestine.

## METHODS

Mature guinea-pig foetuses were used whose weights varied from 50–70 g.; the appearance of the foetuses was very similar to that of the animal after birth. Ten pregnant animals each provided from two to four foetuses. Those which could not be used immediately were destroyed by cutting the carotid arteries and were placed in the refrigerator, to be used when convenient, which was always within four or five hours from death. Preparations made from foetuses which had been frozen for this length of time responded similarly to fresh preparations.

The foetal small intestine varied in length between 16 and 20 in. Segments about 2 in. in length were removed from the required levels of the intestine and suspended in a bath of capacity 100 ml. containing Krebs's physiological salt solution. Four segments taken from different levels of the same intestine were suspended in one bath at the same time, so that simultaneous records were obtained under identical conditions. Drugs were added to the bath in a concentration of $1:2\times10^4$ from a 2-ml. pipette. The concentration of *dl*-noradrenaline in the bath was twice that of *l*-adrenaline when responses to the two drugs were to be compared.

## RESULTS

Fig. 1A shows the responses to adrenaline ($1:1\times10^6$) of segments taken from different levels of the foetal intestine. The terminal ileum contracted; immediately higher segments gave progressively smaller contractions, and at still higher levels the response changed to relaxation. In the region of the duodenum there was a biphasic response with initial relaxation followed by a gradual contraction above the original tonus level. However, some preparations from the foetal duodenum only contracted and others only relaxed. When relaxation occurred, it was usually found that a second dose of adrenaline added to the relaxed tissue cause a contraction. By comparison with the foetal duodenum, the adult duodenum was never found to show anything other than an inhibitory response to adrenaline. The caecum and the colon were invariably relaxed by adrenaline. The whole stomach, on the other hand, was contracted by a similar strength of adrenaline.

Atropine added by itself usually produced a reduction of tone, although this varied in degree between one preparation and another. The responses to adrenaline of intestinal segments after exposure to atropine are shown in Fig. 1B. The contractions of the terminal and immediately

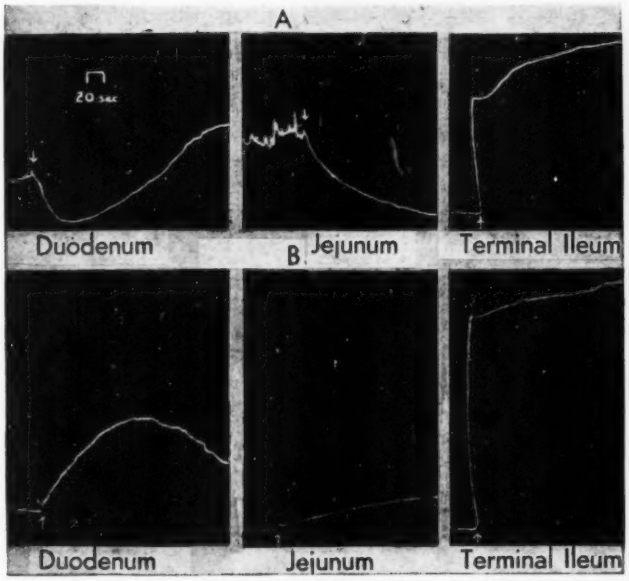


FIG. 1.—The response to adrenaline of isolated segments from different levels of the foetal intestine. (A) Adrenaline ($1:1\times10^6$) was added at the points indicated by the arrows. (B) The responses to adrenaline ($1:1\times10^6$) of the same segments after they had been washed and atropine ($1:7\times10^6$) added to the fresh fluid in the bath.

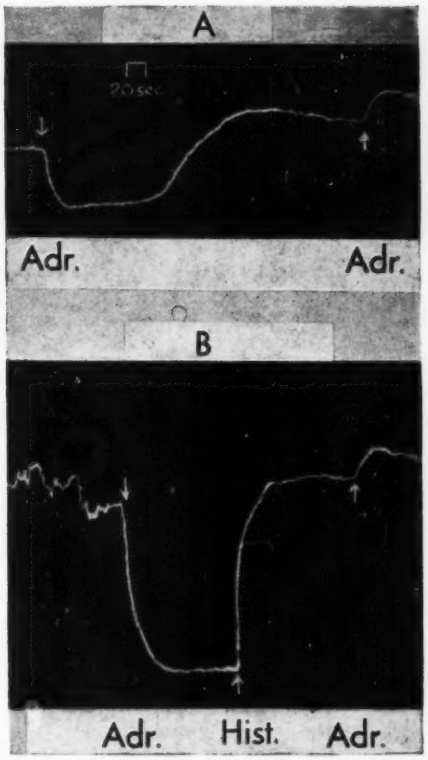


FIG. 2.—The state of contraction and the response of duodenal preparations to adrenaline. (A) The first addition of adrenaline ($1:1\times10^6$) at Adr produced a biphasic response in this preparation; the second addition of adrenaline ($1:1\times10^6$) produced a contraction only. (B) In this preparation the first addition of adrenaline ($1:1\times10^6$) at Adr produced only relaxation. The initial level of tone was regained by the addition of histamine ($1:3\times10^8$) at Hist, and a second dose of adrenaline ($1:1\times10^6$) added at Adr caused only a contraction.

adjacent segments of the ileum were potentiated by atropine, the relaxation of the intermediate segments was changed to a contraction diminishing in strength with their distance from the ileocaecal junction, and the biphasic response of the duodenum became purely motor. On the other hand, atropine did not reverse the inhibitory responses of the foetal large intestine or the adult duodenum to adrenaline.

These changes of response after exposure to atropine are to some extent related to changes in the state of tone. But this is not the whole explanation, as is evident from a consideration of the tracings shown in Fig. 2. Fig. 2A shows a biphasic response by a duodenal segment to adrenaline, but only a contraction when a second dose of adrenaline is added during the motor phase of the first response. In Fig. 2B a duodenal segment relaxed on addition of the first dose of adrenaline, but contracted after the second dose, which was added soon after the original state of tone had been restored by histamine.

Both motor and inhibitory responses were produced by noradrenaline. The response in any segment was similar to that produced by adrenaline, but usually slightly weaker. Adrenaline and noradrenaline each tended to abolish, or more usually reduce, the response to the other. That adrenaline is distinctly more effective in this respect is illustrated by the tracings shown in Fig. 3A and B. The relaxation of a duodenal segment by noradrenaline is cut short by adrenaline, while the relaxation of the same segment by adrenaline is only slightly affected by the equivalent dose of noradrenaline. The effects of adrenaline and noradrenaline on the same segment after it had been exposed to atropine are shown in Fig. 3C and D. Adrenaline is seen here to suppress the motor response to noradrenaline; but the motor response to adrenaline can still be produced in the presence of noradrenaline.

In earlier experiments (Munro, 1951b) employing isolated segments of the terminal ileum of adult guinea-pigs in which the prevailing tone was normally low, it was found that progressively smaller contractions were produced by diminishing the doses of adrenaline, and that a concentration in the region of $1:3\times10^7$ gave a threshold response. In the present experiments using segments of foetal ileum it was occasionally found that a contraction likewise was the only response to adrenaline in a concentration as low as

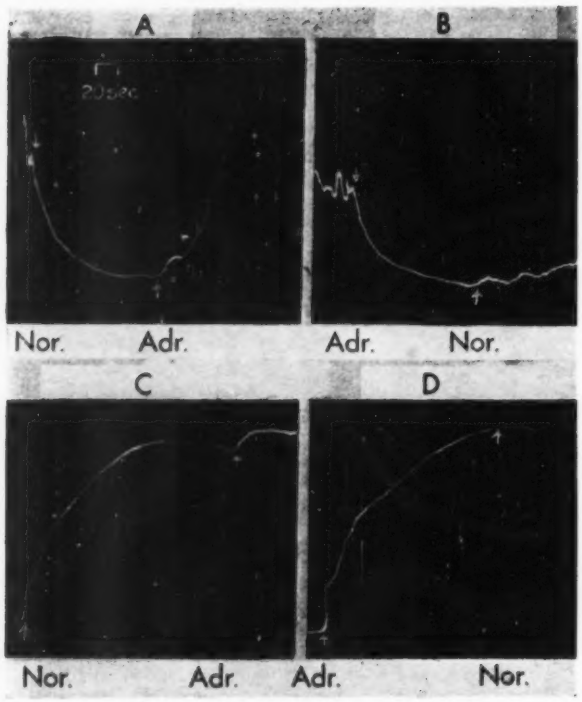


FIG. 3.—The effect of adrenaline on the responses of a duodenal segment to noradrenaline and vice versa. (A) *dl*-Noradrenaline ($1:5\times10^6$) was added at Nor and adrenaline ($1:1\times10^6$) at Adr. (B) The segment was washed and adrenaline ($1:1\times10^6$) was added at Adr and *dl*-noradrenaline ($1:5\times10^6$) at Nor. (C) The same segment was washed and atropine ($1:7\times10^6$) was added 10 min. before the addition of *dl*-noradrenaline ($1:5\times10^6$) at Nor; adrenaline ($1:1\times10^6$) was then added at Adr. (D) The same segment again after washing and the addition of atropine ($1:7\times10^6$). Adrenaline ($1:1\times10^6$) at Adr and *dl*-noradrenaline ($1:5\times10^6$) at Nor.

$1:2\times10^8$. However, it was often possible to show in segments of both foetal and adult terminal ileum, maintained in tone by histamine, that adrenaline in larger doses could produce contraction and in smaller doses relaxation of the same preparation. This is illustrated in a tracing from the isolated terminal ileum of an adult guinea-pig (Fig. 4). Tone was produced by exposure to histamine ($1:3\times10^8$); the addition of adrenaline ($1:7\times10^7$) then relaxed the preparation, but, when the initial tone was regained, the further addition of adrenaline to increase its concentration in the bath by $1:1\times10^6$ produced a strong contraction. Washing the preparation and adding adrenaline ($1:1\times10^6$) again resulted in the segment contracting.

No part of the foetal intestine of the rabbit or the rat showed other than inhibitory responses to adrenaline either before or after exposure to atropine.

## DISCUSSION

In most species adrenaline abolishes the tone and the rhythmicity of the longitudinal musculature of the small intestine. On the other hand, it contracts the sphincters, e.g., the ileo-caecal (Elliott, 1904). The fact that the duodenum and a variable length of the terminal part of the ileum are the parts of the small intestine which are contracted by adrenaline in the foetal guinea-pig rather suggests that in the foetus differentiation of function between sphincter and duodenal muscle (both of similar morphological origin) is as yet incomplete. But it is interesting that the motor component of the response to adrenaline should disappear from the duodenum during the further development of the animal, whilst it persists in the terminal part of the ileum. The motor response of the duodenum is at no stage so strong as that of the terminal ileum. A comparison of the contraction produced by adrenaline in foetal and adult segments shows that in the former it is usually more gradual in onset and more sustained. The foetal tracing is typically humpbacked with the initial rapid phase of the contraction relatively small in duodenal segments and large in segments from the terminal ileum.

The question arises whether these motor responses to adrenaline play any part in normal

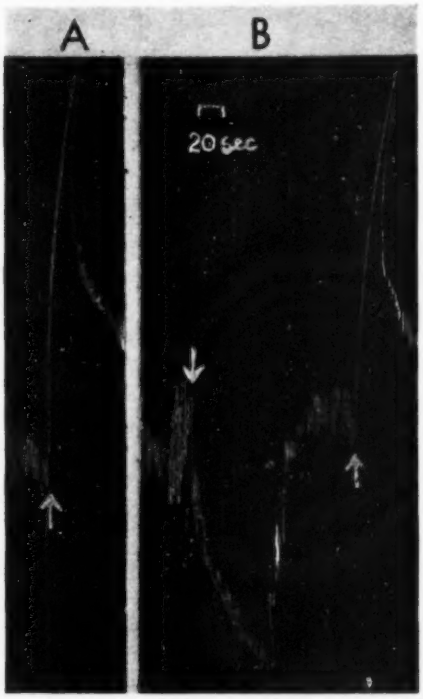


FIG. 4.—The relation between the dose of adrenaline and the type of the response. The preparation was taken from the terminal part of the ileum of an adult animal, and was maintained in moderate tone by the addition of histamine ($1:3\times10^8$) to the bath fluid. (A) Adrenaline ($1:1\times10^6$) was added at the point indicated by the arrow. (B) The same segment after washing and the addition of histamine as before. At the first arrow adrenaline ($1:7\times10^7$) was added; at the second arrow adrenaline ($1:1\times10^6$) was added.

function, either in the foetus or the adult. Since there is usually a reversal of the motor response in isolated preparations when the dose of adrenaline is sufficiently reduced, it appears likely that only relaxation would result from the concentration of adrenaline normally occurring in the circulating blood. Dale and Richards (1918) have described a similar type of reversal in the response of the blood vessels to adrenaline. A sufficiently great diminution in the dose of adrenaline may also change the response of the longitudinal muscle of the isolated rabbit ileum (Hoskins, 1912), but in this preparation it is the higher concentration of adrenaline which causes relaxation.

It would appear that the prevailing tone of the preparation has some effect in determining whether the response to adrenaline will be motor or inhibitory, but this is clearly not the only factor of importance. A single dose of adrenaline applied to a duodenal segment usually produces a biphasic response, but when successive doses of adrenaline are added to such a preparation without intervening washing, it is only the first dose which produces a response with the inhibitory component present. Adrenaline would thus appear to attach itself preferentially to the inhibitory receptors and to saturate them more readily than the motor receptors.

No great difference was observed in the strength of either the motor or the inhibitory responses of foetal intestinal preparations to equivalent concentrations of adrenaline and noradrenaline. Nevertheless, the observations showed that their action was not identical. Adrenaline was able to overcome the inhibitory response to noradrenaline, and, in those experiments in which the response to noradrenaline was motor, to produce a superimposed contraction. Noradrenaline, on the other hand, was much less effective in modifying the responses, either motor or inhibitory, to adrenaline. If the difference were due to more rapid destruction of noradrenaline than of adrenaline it would be expected that the response of the preparation to noradrenaline would be less sustained than that to adrenaline. Since the responses were similar in this respect, some other explanation must be sought for the difference between the two drugs.

## Summary

1. Adrenaline and noradrenaline caused contraction of isolated segments taken from the two ends of the small intestine of the foetal guinea-pig The intervening region was either relaxed or not affected. Atropine potentiated the adrenaline motor response and reversed the adrenaline inhibitory response.

2. A dual response to adrenaline has been demonstrated in segments from both foetal and adult terminal ileum. Larger doses cause contraction and smaller doses relaxation.

3. Adrenaline reduces or abolishes both the contraction and relaxation caused by noradrenaline in segments from the foetal ileum.

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# THE EFFECT OF COPPER ON THE ENZYMIC OXIDATION OF HISTAMINE AND ALIPHATIC DIAMINES

BY

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The mechanism of oxidation of histamine by histaminase is not yet fully understood. Zeller (1938) described the enzymic oxidation of short-chain diamines, such as putrescine and cadaverine, and suggested that this reaction was catalysed by histaminase; the reaction was considered to be an oxidative removal of the amino-group with the formation of the corresponding aldehyde. For histamine this aldehyde would be imidazoleacetaldehyde.

There are indications that the enzymic oxidation of putrescine and cadaverine differs in certain respects from that of histamine. According to Kapeller-Adler (1949), both dialysis and addition of flavine-adenine dinucleotide (FAD) have different effects on the enzymic reaction according to whether the substrate is histamine or an aliphatic diamine. Differences in the action of steroid hormones and gonadotrophins on the enzymic oxidation of histamine on the one hand and of cadaverine on the other have also been described (Kapeller-Adler, 1951). Tabor (1951), using a purified preparation of histaminase from pig's kidney, has obtained evidence that aldehyde oxidase will oxidize the primary product of the enzymic oxidation of histamine but not of cadaverine.

Holmberg and Laurell (1948) reported that blood plasma contains a copper-protein which actively destroys histamine, but this has not been confirmed (see Zeller, 1951). The effect of copper on the enzymic oxidation of histamine by aqueous extracts of human placenta has recently been examined (Bruns and Stüttgen, 1951). Oxygen was found to be taken up when copper was added to preparations of histaminase plus histamine even after the histaminase reaction had been completed.

In the present work the action of copper on the enzymic oxidation of both histamine and aliphatic diamines by a preparation of pig kidney has been studied. A few experiments on the action of other metals will also be reported.

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## METHODS

*Enzyme Preparations.*—The starting material in all experiments was an extract of acetone-dried powder of pig's kidney. The kidneys were usually frozen at $-10°$ C. overnight and then worked up with acetone. The powder thus obtained was extracted with 0.067 M sodium phosphate buffer, *p*H 7.4, 10 ml. of buffer to each g. of powder. The extract was stirred and left standing for 1 hour and then centrifuged. The sediment was washed with more buffer and the second supernatant was added to the first.

This crude extract was fractionated according to Tabor (1951). In this procedure the enzyme is precipitated when the concentration of $Na_2SO_4$ is increased from 14.5 to 21.5% (w/v). The precipitate was dissolved in a small volume of 0.067 M sodium phosphate buffer, *p*H 7.4, and dialysed against running tap water for 1 hour. One-half volume of 0.2 M sodium phosphate buffer *p*H 7.4 was added. This solution contained all the activity of the crude extract and the purification on the basis of non-dialysable nitrogen was about fourfold. This material was used in most of the experiments.

*Manometric Experiments.*—1.6 ml. of the enzyme solution was used in the main compartment of the manometer flask. 0.2 ml. of water or $5\times10^{-2}$ M substrate solution was in the side bulb. The side bulb also contained 0.2 ml. water or 0.2 ml. of a solution of a metal salt, e.g., $CuSO_4$. The total volume was 2 ml. The central tube contained 0.3 ml. N KOH. The gas phase was oxygen and the temperature 37.5° C.

## RESULTS

When histamine was incubated with histaminase in the presence of copper as $CuSO_4$ an increase in the rate of oxygen consumption was regularly observed. With cadaverine as substrate there was no similar increase and the rate of oxidation in the presence of copper was often slightly decreased. A typical experiment is reproduced in Fig. 1, in which the rate of oxygen uptake in the presence of histamine was approximately doubled by the addition of $1.5\times10^{-3}$ M $CuSO_4$, whereas the copper salt had no similar effect upon the rate of oxidation of cadaverine.

With histamine as substrate the increase in the rate of oxygen consumption was greatest when

the concentration of $CuSO_4$ was between 2 and $5 \times 10^{-3}$ M. With cadaverine the oxygen consumption was not increased by $CuSO_4$ at any concentration. The only effect of copper on the rate of oxidation was a decrease. This decrease was about 50% in $5 \times 10^{-3}$ M $CuSO_4$. These results are shown in Fig. 2. In subsequent experiments the concentration of copper used was $1.5 \times 10^{-3}$ M. At that concentration the increase in the rate of oxygen consumption with histamine was almost maximal, whereas the oxidation of cadaverine was slowed only slightly.

It was necessary to find out whether the increased consumption of oxygen in the presence of copper was accompanied by a similar increase in the rate of inactivation of histamine. Histamine was therefore incubated with histaminase in the presence and absence of copper in a number of manometer flasks. While the oxidation proceeded, the histamine remaining in the flasks was determined by biological assay.

Six manometers were set up. Three of the flasks contained copper and three did not. The

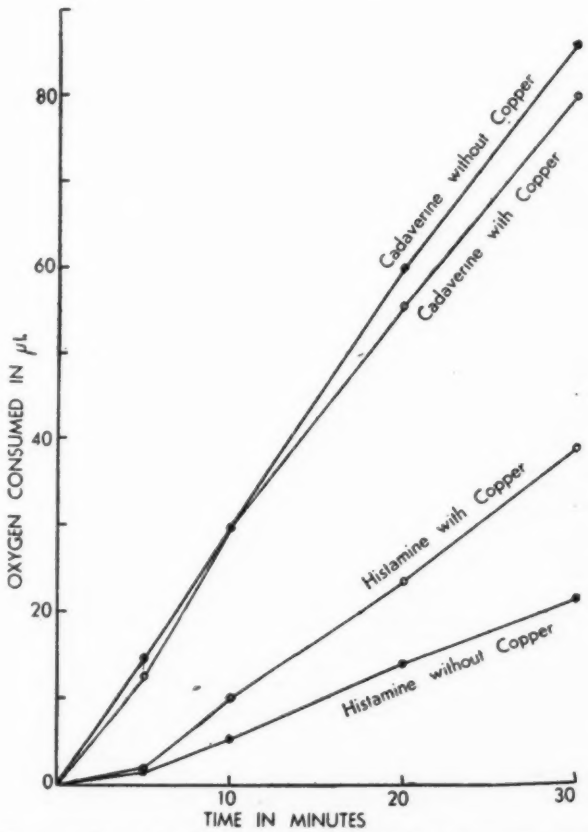


FIG. 1.—Effect of $CuSO_4$ on the enzymic oxidation of histamine and cadaverine. Abscissa: time in minutes. Ordinate: oxygen consumed in excess of blank. Initial substrate concentration: $5 \times 10^{-3}$M. Concentration of $CuSO_4$: $1.5 \times 10^{-3}$M.

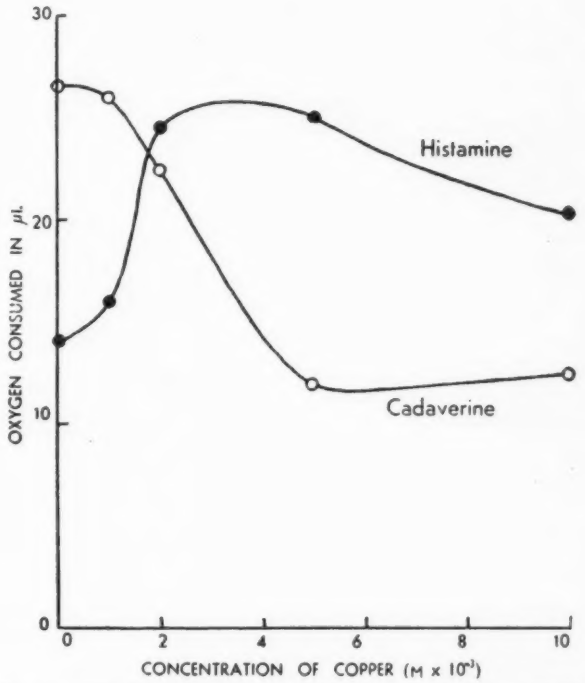


FIG. 2.—Effect of different concentrations of $CuSO_4$ on oxygen consumption with histamine and cadaverine. Initial substrate concentration: $5 \times 10^{-3}$M.

initial concentration of $CuSO_4$ was $5 \times 10^{-3}$ M and the concentration of $CuSO_4$ was $1.5 \times 10^{-3}$ M. After 0, 90, and 240 minutes the incubation of one pair of flasks, one without copper and one with copper, was terminated. One ml. of the content was withdrawn and added to 1 ml. of 12.5% (w/v) trichloracetic acid. The mixture was filtered and 1 ml. of the filtrate was prepared for histamine assay by Code's method (1937). The procedure differed from Code's procedure in that the samples were not boiled but heated on the boiling water bath. The histamine assay was carried out on the isolated ileum of the guinea-pig. The results are set out in Table I. They showed clearly that in spite of the different amounts of oxygen consumed the rate of inactivation of histamine was the same in the presence and absence of copper. This meant that the additional oxygen taken up in the presence of copper was used in an oxidation reaction other than the oxidation of histamine by histaminase.

This interpretation was supported by experiments in which copper was added when the oxygen uptake in the histaminase reaction had ceased. The result of such an experiment is shown in Fig. 3. In this experiment the histaminase reaction came to a standstill with the uptake of 71 μl. of oxygen. This is 1.27 atoms of oxygen per mole of added histamine. Copper was then added from a second

TABLE I
THE RATES OF INACTIVATION OF HISTAMINE BY HISTAMINASE IN THE ABSENCE AND IN THE PRESENCE OF COPPER

| Time (min.) | Oxygen Consumed (μl.) | | Total Mean Histamine in Flask (μg.) | | |
|---|---|---|---|---|---|
| | Without $CuSO_4$ | With $CuSO_4$ ($1.5\times10^{-3}$M) | Calculated from Oxygen Consumption | Without $CuSO_4$ Found | With $CuSO_4$ ($1.3\times10^{-3}$M) Found |
| 0 | 0 | 0 | 1,110 | 972 | 1,075 |
| 90 | 46 | 126 | 655 | 590 | 618 |
| 240 | 121 | 268 | 0 | 0 | 43 |

side bulb of the manometer flask. This caused a further rapid uptake of oxygen until the total consumption was approximately doubled. This result agrees with similar observations of Bruns and Stüttgen (1951) on extracts of human placenta.

*Effect of Copper on the Enzymic Oxidation of Aliphatic Diamines*

With cadaverine as substrate, copper did not increase the rate of oxygen uptake. Also, when copper was added after the enzymic oxidation of cadaverine had come to a standstill, no further oxygen uptake occurred. This experiment was repeated with other diamines known to be oxidized by the pig's kidney preparation: trimethylene diamine, tetramethylene diamine, hexamethylene diamine, heptamethylene diamine, and octamethylene diamine.

In each experiment the diamine was added at zero time and when the uptake of oxygen due to the oxidation of the diamine had come to a standstill $CuSO_4$ was added from the side bulb. As expected, all the diamines from $C_3$ to $C_8$ were oxidized ; but the addition of copper did not cause oxidation to be resumed. An experiment with octamethylene diamine is shown in Fig. 4. The oxygen uptake was followed until it came to a standstill when one atom per mole of substrate had been taken up. In one flask, $CuSO_4$ was then added without causing any uptake of oxygen. In another flask putrescine was added. This caused a renewed oxidation, indicating that the enzyme was still active.

Agmatine ($NH_2C(:NH)NH(CH_2)_4NH_2$) was also oxidized by the preparation. Addition of $CuSO_4$ did not cause a renewed oxygen uptake.

A few salts of other metals were also tested with histamine as substrate and it was found that cobalt caused an increased rate of oxygen consumption. This is shown in Fig. 5, where the effect of $1.5\times10^{-3}$ M $CoCl_2$ was compared with that of $1.5\times10^{-3}$ M $CuSO_4$. $FeCl_3$, $ZnSO_4$, and $MnCl_2$ at the same concentration slightly reduced the oxygen consumption.

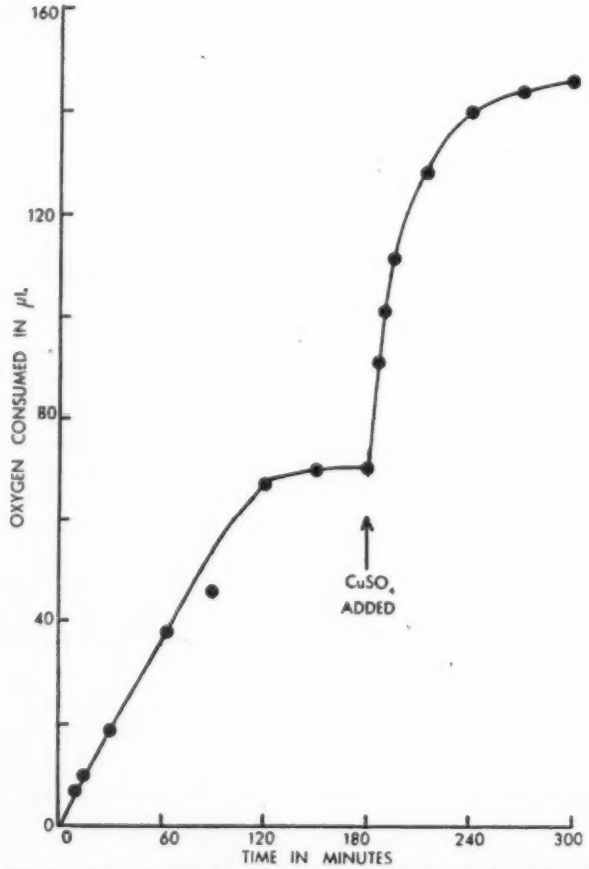


FIG. 3.—Enzymic oxidation of histamine and subsequent addition of $CuSO_4$. $1.5\times10^{-3}$M $CuSO_4$ added after 180 min

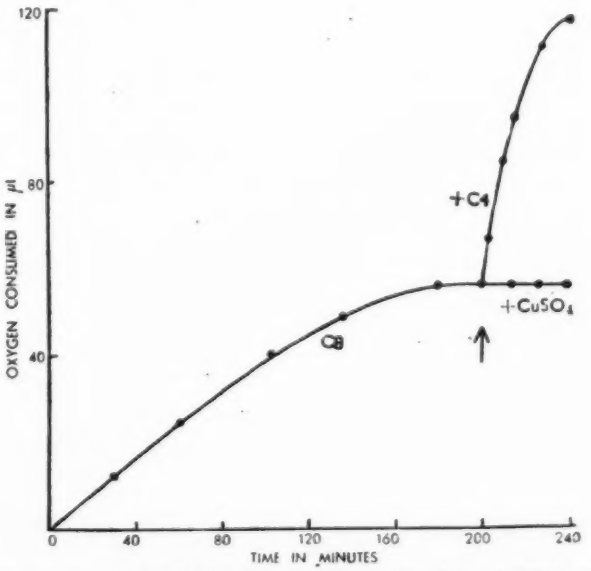


FIG. 4.—Enzymic oxidation of octamethylene diamine and effects of subsequent addition of (*a*) $CuSO_4$ and (*b*) putrescine ($C_4$). Initial substrate concentration: $2.5\times10^{-3}$M. Concentration of $CuSO_4$: $1.5\times10^{-3}$M. Concentration of putrescine: $2.27\times10^{-3}$M.

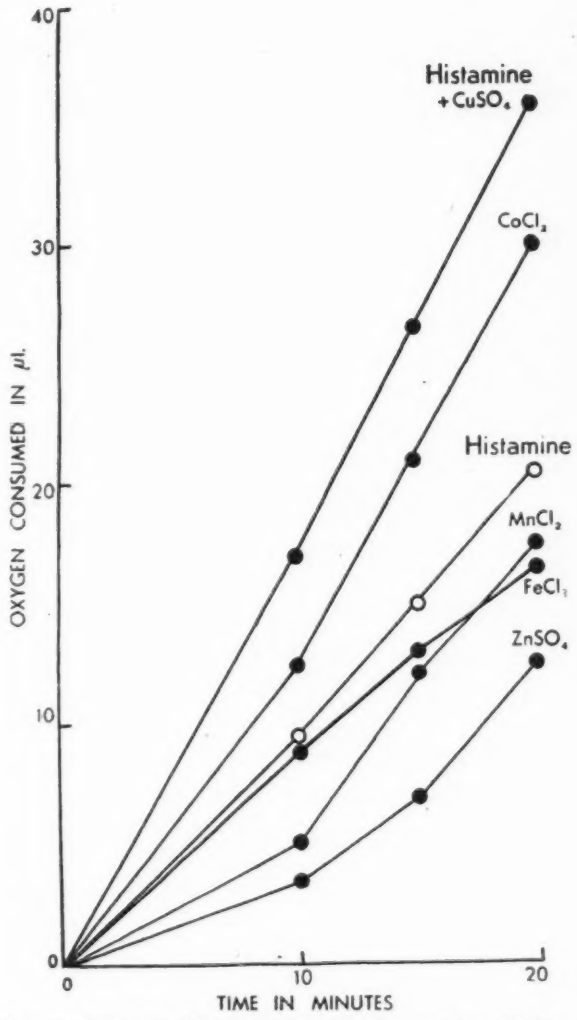


FIG. 5.—Effect of different metals on the enzymic oxidation of histamine. Initial substrate concentration: $5\times10^{-3}$M. Concentration of metal: $1.5\times10^{-3}$M.

## DISCUSSION

The experiments with histamine show clearly that the increased oxygen uptake in the presence of copper is not due to an oxidation of histamine but of a product of its primary oxidation by histaminase. This is in complete agreement with the observations of Bruns and Stüttgen (1951) on human placenta.

There is still some doubt about the initial point of attack by histaminase on the histamine molecule, but the suggestion that imidazoleacetaldehyde is the first oxidation product has received support from Tabor's (1951) observation that aldehyde oxidase causes a further oxidation after the histaminase reaction is over. Thus it seems that copper catalyses the same reaction as aldehyde oxidase, i.e. the formation of imidazoleacetic acid from imidazoleacetaldehyde.

Copper did not cause an additional oxygen uptake when the aliphatic diamines were the substrates. This is analogous to the observation by Tabor (1951) that the aldehyde oxidase does not act on the primary products formed in the enzymic oxidation of putrescine and cadaverine. He suggests that this is due to the cyclization of the ω-amino-aldehydes formed. Cyclization would probably occur less readily with the shorter and longer amino-aldehydes. It is interesting, therefore, that all the diamines tested gave rise to oxidation products which were not further oxidized by copper.

These observations show that differences in the rates of oxygen uptake when histamine is oxidized by histaminase do not always indicate that the inactivation of histamine proceeds at a different rate. They also show that differences in the oxidation of histamine and aliphatic diamines need not be explained by assuming that two different enzymes are concerned unless differences in the secondary reactions of the products of the enzymic reaction are excluded.

## SUMMARY

When the oxidation of histamine is catalysed by histaminase prepared from pig's kidney, the presence of $1.5\times10^{-3}$ M copper increases the rate of oxygen consumption, but the rate of inactivation of histamine is not increased. When copper is added after the histaminase reaction has stopped there is an additional uptake of about one atom of oxygen per molecule of histamine. Copper has no similar effect when aliphatic diamines are oxidized by the pig kidney preparation. The evidence suggests that copper catalyses the further oxidation of the primary oxidation product of histamine but not that of the aliphatic diamines.

I would like to thank Professor J. H. Burn, F.R.S., for his hospitality and interest, and Dr. H. Blaschko for help and advice. I am grateful to Miss Jean Himms for help in these experiments.

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# THE MEASUREMENT OF THE INFLUENCE OF DRUGS ON VOLUNTARY ACTIVITY IN MICE

BY

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The purpose of the present communication is to describe a method which distinguishes qualitatively between co-ordinated running or walking movements and twitching or convulsive movements in mice, and which yields quantitative information on the former. Winter and Flataker (1951) have used a method very similar to the one to be described in semi-quantitative studies on rats, and Waterman (1947) has briefly reviewed several procedures in common practice.

## METHOD

The apparatus consists of a rectangular cage with wooden floor and ends and transparent plastic sides. A beam of light is passed across the cage along the short axis, through the two transparent sides, and on to a photoelectric cell which is adjusted so that when a mouse breaks the beam of light the cell activates a magnetic digital counter. The cage is 42 cm. long, 23.5 cm. wide, and the sides were 8.5 cm. high. These precise dimensions are not critical.

Male albino mice of Carworth Farms CF-1 strain were used throughout. They were allowed free access to food and water until the time they were put in the experimental cage.

Drugs were dissolved in 0.9% sodium chloride solution and were injected intraperitoneally in graded doses to groups of five mice. Dosages of drugs are expressed in terms of drug base. Control groups received 0.9% sodium chloride solution only. Immediately after injection the five mice were put together into the cage, and the number of times the light beam was broken (henceforth called the "count") in 15 minutes was noted.

## RESULTS

Some idea of the reproducibility of the method may be obtained by inspection of the figures for the saline controls in Table I. Each of these figures is the mean of two groups of five mice.

A series of 19 groups of five mice injected with saline were observed over a period of about one month (July–August, 1949). The mean count was 132; the standard deviation of a single count was 19, giving a coefficient of variation $\left(\frac{\text{S.D.}}{\text{mean}} \times 100\right)$ of 15%. There was no evidence of any consistent trend in the counts during the time. During the same period, 11 groups of mice were observed after injection of 1.25 mg./kg. of methamphetamine. The mean count was 325, with a standard deviation of 89, giving a coefficient of variation of 27%. When the responses to methamphetamine were expressed as the ratio of

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TABLE I

THE EFFECT OF VARIOUS DRUGS ON CO-ORDINATED ACTIVITY IN MICE

| Dose mg./kg. | Epinephrine C | Epinephrine R | Methamphetamine C | Methamphetamine R | Strychnine C | Strychnine R | Nicotine C | Nicotine R | Picrotoxin C | Picrotoxin R | Cocaine C | Cocaine R | Amphetamine C | Amphetamine R | Ephedrine C | Ephedrine R | Caffeine C | Caffeine R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Saline only | 133 | 1·00 | 139 | 1·00 | 133 | 1·00 | 149 | 1·00 | 133 | 1·00 | 129 | 1·00 | 115 | 1·00 | 111 | 1·00 | 131 | 1·00 |
| 0·0195 | 131 | 0·98 | | | | | | | | | | | | | | | | |
| 0·039 | 107 | 0·80 | 149 | 1·07 | 130 | 0·97 | | | | | | | | | | | | |
| 0·078 | 111 | 0·83 | 88 | 0·63 | 190 | 1·42 | | | | | | | | | | | | |
| 0·156 | 39 | 0·29 | 183 | 1·32 | 142 | 1·06 | 123 | 0·82 | 130 | 0·98 | | | | | 108 | 0·97 | | |
| 0·312 | 42 | 0·31 | 178 | 1·28 | 124 | 0·93 | 169 | 1·13 | 113 | 0·85 | 139 | 0·95 | | | 109 | 0·98 | | |
| 0·625 | 55 | 0·41 | 224 | 1·62 | 122 | 0·91 | 133 | 0·89 | 127 | 0·95 | 164 | 1·12 | 199 | 1·72 | 132 | 1·19 | | |
| 1·25 | 15 | 0·11 | 367 | 2·64 | 66 | 0·49 | 7 | 0·05 | 11 | 0·08 | 164 | 1·12 | 152 | 1·30 | 165 | 1·49 | | |
| 2·50 | 3 | 0·02 | 312 | 2·24 | 37 | 0·28 | 14 | 0·09 | 7 | 0·05 | 193 | 1·40 | 181 | 1·57 | 88 | 0·79 | 203 | 1·54 |
| 5·0 | | | 386 | 2·78 | | | 14 | 0·09 | 13 | 0·10 | 190 | 1·37 | 309 | 2·68 | 62 | 0·56 | 165 | 1·25 |
| 10·0 | | | 232 | 1·67 | | | | | 26 | 0·20 | 317 | 2·31 | 356 | 3·09 | 35 | 0·32 | 197 | 1·45 |
| 20·0 | | | 195 | 1·41 | | | | | | | 400 | 2·92 | 265 | 2·29 | 74 | 0·67 | 225 | 1·71 |
| 40·0 | | | 96 | 0·69 | | | | | | | 639 | 4·63 | 109 | 0·94 | 37 | 0·33 | 220 | 1·67 |
| 80·0 | | | | | | | | | | | 158 | 1·17 | 38 | 0·33 | 35 | 0·32 | 72 | 0·61 |
| 160·0 | | | | | | | | | | | | | | | 73 | 0·66 | | |

C: Number of counts in 15 minutes. R: Ratio of counts after drug to count of saline controls on same day.

the count after methamphetamine to the count of the controls on the same day, the 11 groups gave a mean ratio of 2.49 and the coefficient of variation was 28%. The ratios have been used as ordinates in plotting the dose response curves shown in Fig. 1.

Inspection of Fig. 1 shows that the central nervous stimulant drugs studied may be divided into two groups according to the form of the dose response curve obtained by this method. *Group 1 :* Drugs which, at some dose level, caused an increase in the co-ordinated activity of the mice. This group includes caffeine, cocaine, ephedrine, amphetamine, and methamphetamine. *Group 2 :* Drugs which caused no significant increase in co-ordinated activity in the mice at any dose level. Picrotoxin, nicotine, and strychnine fall into this group, as also do procaine and pyrilamine (not shown in Fig. 1).

It will be noted that the drugs of group 1 are known clinically to prevent sleep and to cause euphoria in man. Evidence of central nervous stimulation by drugs of group 2 was seen with the larger doses in the form of twitchings and convulsions in some mice.

TABLE II

METHODS OF COMPARING THE ACTIVITY OF COCAINE AND EPHEDRINE RELATIVE TO THAT OF METHAMPHETAMINE=1.

(From data of Table I and Fig. 1)

| Basis of Comparison | Cocaine | Ephedrine |
|---|---|---|
| Reciprocal of doses causing 50% increase in activity over controls .. | 0·091 | 0·36 |
| Reciprocal of doses causing 100% increase in activity over controls .. | 0·1 | — |
| Magnitude of effect caused by most active dose .. .. .. .. | 1·6 | 0·53 |
| Area under dose response curve above control level up to most active dose .. | 1·3 | 0·12 |

The dose response curves of all the drugs of group 1 showed a peak. In other words, there was a dose level for each drug which caused maximum co-ordinated activity ; doses higher than this, though usually still well below the lethal range, led to less activity. For example, the maximum activity following methamphetamine was obtained with a dose of 5 mg./kg. Higher doses caused less activity, although the LD50 under these conditions was about 100 mg./kg. and only two out of 20 mice were killed by 80 mg./kg.

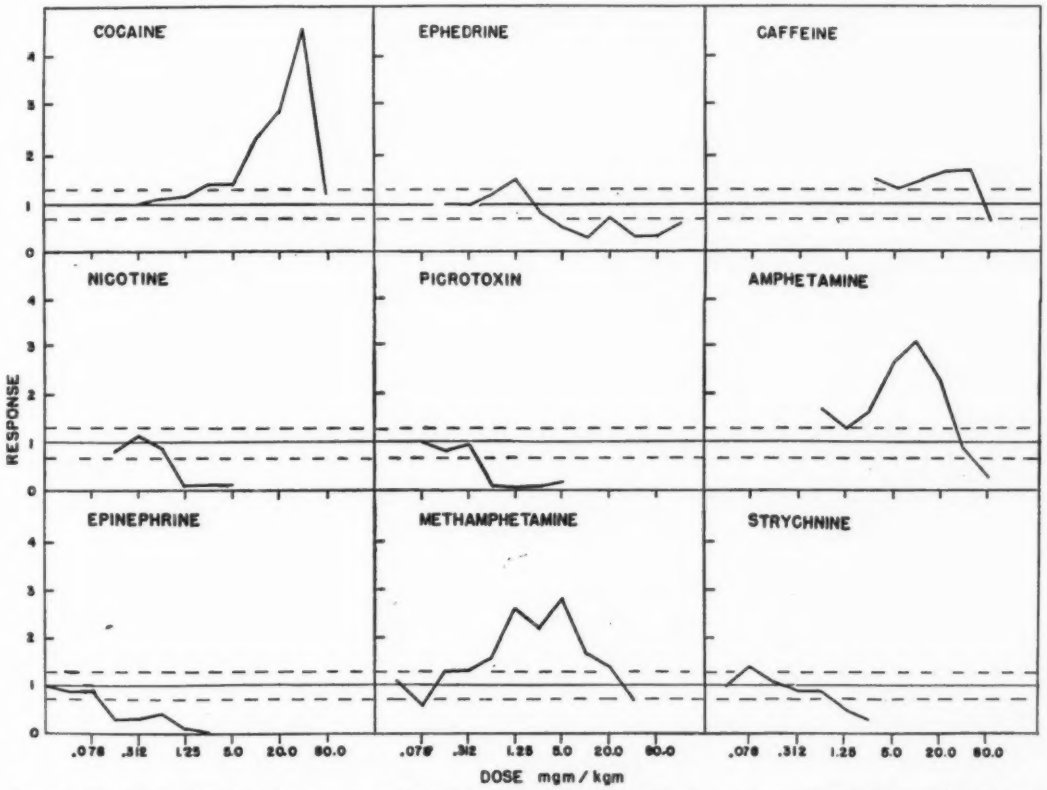


FIG. 1.—The effect of various drugs on co-ordinated activity of mice. Ordinate—response expressed as ratio of count after drug to count of controls on same day. Two dotted lines indicate ± two standard deviations of control counts over whole period of observation.

The method gives quantitative information on the action of drugs of group 1. Table II illustrates some ways in which the relative activities of such drugs may be compared.

## Discussion

Obviously only walking or running activity which leads to a mouse breaking the light beam is measured by the apparatus; similar activity confined to the ends of the cage is not detected. However, it seems reasonable to expect that the number of times the beam is broken will be proportional to the total amount of movement of translation of the mice. A larger proportion of the total activity could be measured directly by reflecting the beam across the cage one or more times before it reaches the photo cell; such an arrangement was tried, but resulted in no real advantage.

The counts given by groups of five mice showed a smaller coefficient of variation than those given by groups larger or smaller than five. When more than one mouse is in the cage at the same time, the activity of any one mouse is affected by the activity of the others. In practice, the net effect of such interactions has been to reduce the variability of the counts of groups of five mice in the cage simultaneously below that found for the totals or means of five mice put in the cage consecutively.

The rate of counting of groups of control mice was found to remain quite constant for the first 15–20 minutes after their introduction into the cage. Subsequently, the rate of counting decreased, sometimes steeply. Under the conditions used, counts taken for a period of 15 minutes showed a smaller coefficient of variation than those obtained with any other reasonable period.

Sufficiently large doses of the drugs of group 1 led to a marked decrease in co-ordinated movements around the cage and the production of twitchings and convulsions. These effects were certainly the main reason for the fall in measured activity. It is interesting to note that Schulte, Tainter, and Dille (1939) found that their "jiggle cage" indicated little increase in activity in rats with subconvulsive doses of picrotoxin or metrazole. However, convulsive doses led to a considerable increase in measured total activity. The method here described distinguishes more clearly, at least with the drugs studied, between central nervous stimulating drugs which are used as "psychomotor stimulants" and those which are either used for the production of convulsions or counteraction of states of central nervous depression, or whose central nervous actions are usually unsought side-effects. It is suggested that the test might be useful in the search for better drugs of the former kind.

For screening purposes it is suggested that the testing of drugs at two dose levels at 10% and 50% of their LD50 will give maximum economy with little risk of missing potentially useful drugs. For definitive evaluation of a drug, construction of the whole dose response curve is necessary; if from this a single numerical index of activity is required, the area under the dose response curve above control level and up to the most active dose summarizes most information. Experience to date indicates that, using mice of the same strain and sex, it is not necessary to repeat the whole dose response curve for the standard drug every time such an evaluation is performed.

## Summary

A method of measuring running and walking activity in mice by counting the number of times they interrupt a beam of light has been described. It has been found that psychomotor stimulants such as cocaine and methamphetamine cause a great increase in such activity, while other central nervous stimulant drugs such as picrotoxin and strychnine cause no change or a decrease in activity.

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# THE PHARMACOLOGICAL EFFECTS OF MASSIVE DOSES OF NICOTINAMIDE

BY

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Nicotinic acid and its derivatives are natural components of foodstuffs and of a number of intracellular enzymes. They are not very toxic and in the usual therapeutic doses produce no significant pharmacological effects. The transient nitrite-like vasodilatation caused by nicotinic acid is well known (Smith, Ruffen, and Smith, 1937; Robertson, 1941). Since nicotinamide does not produce this side-effect it is preferred for therapeutic purposes. However, on injection of massive doses of nicotinamide we observed certain characteristic phenomena which are described in this paper.

## METHODS

Cats, dogs, and rabbits were anaesthetized with pentobarbitone sodium (35 mg./kg. intraperitoneally), if necessary after ether, but ether induction was not used in those experiments in which blood sugar determinations were done. Respiratory movements were recorded by connecting a tambour to a T-shaped tracheal cannula. Carotid arterial pressure was recorded with a mercury manometer. For glucose determinations blood samples in these animals were withdrawn from the femoral arteries and analysed by the method of Hagedorn-Jensen. Urine was collected by insertion of ureteral cannulae in dogs and by vesical cannulae in cats. When rabbits were used without anaesthesia, blood samples were taken from the ear vein for sugar determination and from the heart for estimation of nicotinamide.

For the diuresis experiments groups of four rats of 150–250 g. were fasted for 16 hours, but were allowed water until the start of the experiment.

Nicotinamide was determined colorimetrically as the ferric chloride complex of nicotinohydroxamic acid (Bergmann, 1952). This method is specific for the amide and those of its derivatives which still contain the amide group, e.g., the $N^1$-methyl derivative cannot be distinguished from nicotinamide, but nicotinic *acid* gives a negative response in the test. When nicotinamide is added to whole blood, deproteinization with 10% trichloroacetic acid (Procedure 1) gives a colorimetric value of 45 Klett units (KU)/$\mu$mole/ml. This method has the disadvantage that trichloroacetic acid itself reacts slowly with alkaline hydroxylamine, so that a blank without blood must be run simultaneously in every experiment.

Deproteinization with the Hagedorn-Jensen reagent (Procedure 2) leads to a value of about 55 KU/$\mu$mole/ml. The reason for the discrepancy between the two methods is not yet clear and is being studied further. In both procedures the linear relationship between concentration of nicotinamide and extinction has been established. Blood usually gives a blank reading of 240 KU/ml., and urine of 100–150. Occasionally, however, we observed blank readings for urine of 300 KU/ml. and higher. The nature of the chromogenic material in urine is now under investigation, since it is improbable that such large normal blank values are due to the excretion of nicotinamide derivatives.

*Procedure 1:* 0.5 ml. whole blood is added to 1 ml. of 10% trichloroacetic acid. After standing at room temperature for 15 minutes the tube is centrifuged, and an aliquot of the supernatant (usually 0.75 ml.) is pipetted off. Water is added to give 1 ml. and then 2 ml. of the alkaline hydroxylamine reagent (made by mixing 50 ml. of 2 N hydroxylamine sulphate and 55 ml. of 3.5 N NaOH). The test-tube is left in a water bath at 26° C. for 12–16 hours. Then 1 ml. of 3.5 N HCl is added, the mixture swirled well, and the coloured complex formed by addition of 1 ml. of 0.74 M $FeCl_3$ dissolved in 0.1 N HCl. Readings are taken within 10 minutes. If the reading exceeds 300 KU the solution is diluted with 0.37 M $FeCl_3$ dissolved in 0.1 N HCl.

*Procedure 2:* 0.5 ml. whole blood is added to 2.5 ml. of a mixture prepared from 5 volumes of 0.45% $ZnSO_4.7H_2O$ and one volume of 0.1 N NaOH. The test-tube is placed in a boiling-water bath for four minutes, then centrifuged, and 1 ml. of the supernatant is added to 2 ml. of alkaline hydroxylamine reagent (prepared from *equal* volumes of the two components). The mixture is treated further as above.

Nicotinamide in urine is determined directly on 1 ml. or a fraction of it. The hydroxylamine solution is made up as in Procedure 2. Urine from the pre-experimental period is used as blank.

## Results

*Toxicity of Nicotinamide.*—The LD50 was determined graphically from the dose-response curve, 20 animals being used for each dose. We find for mice values similar to those obtained by earlier investigators; LD50 after intravenous injection 1.62 g./kg.; LD50 after intraperitoneal injection 1.80 g./kg. The closeness of these two values and of that for the subcutaneous LD50, found by Brazda and Coulson (1946) to be 1.68 g./kg., indicates that nicotinamide is rapidly absorbed into the circulating blood. This conclusion finds support in the experiments reported below. Death usually occurs within 6–12 hours. After large doses the animals first show pronounced tachypnoea, and later prostration and shallow respiration. The toxicity is not altered by intraperitoneal administration of 1.0 g./kg. *d, l*-methionine one hour before the injection of nicotinamide, although death is somewhat delayed.

The toxicity of nicotinamide is much higher for hypophysectomized rats, which succumb to doses of 0.7–0.8 g./kg. intraperitoneally.

*Effect on Blood Pressure, Respiration, and Heart Rate.*—For these experiments 9 dogs, 12 cats, and 4 rabbits were used. Intravenous injection of 0.5–1.0 g./kg. of nicotinamide produces a sudden fall of blood pressure which reaches a minimum within less than 1 minute (Fig. 1). Then gradual recovery begins; it usually takes 10–20 minutes before a constant level is reached again. In cats little change of heart rate is observed, whereas dogs react with a marked tachycardia (e.g. from 168 to 240/min.). The respiratory movements may stop for the first few seconds, to continue with increased depth and sometimes with increased frequency, so that the pulmonary ventilation is always increased. These effects are also observed after cutting both vagi. The spinal cat shows a corresponding, though less pronounced, fall of blood pressure. The blood pressure and respiratory effects are also obtained after intraperitoneal injection of 0.5–1.0 g./kg. nicotinamide, although they are less abrupt. This shows that the substance is absorbed quickly from the peritoneal cavity, an observation which agrees with those mentioned above in which the LD50 i.p. was found to be very close to the LD50 i.v.

If the blood pressure is raised by intravenous injection of *l*-ephedrine (0.8 mg./kg.), subsequent injection of nicotinamide causes a pronounced fall of blood pressure. *l*-Epinephrine (4 μg./kg.) causes a temporary rise when injected into a cat at the time when the blood pressure has reached its minimum after administration of nicotinamide.

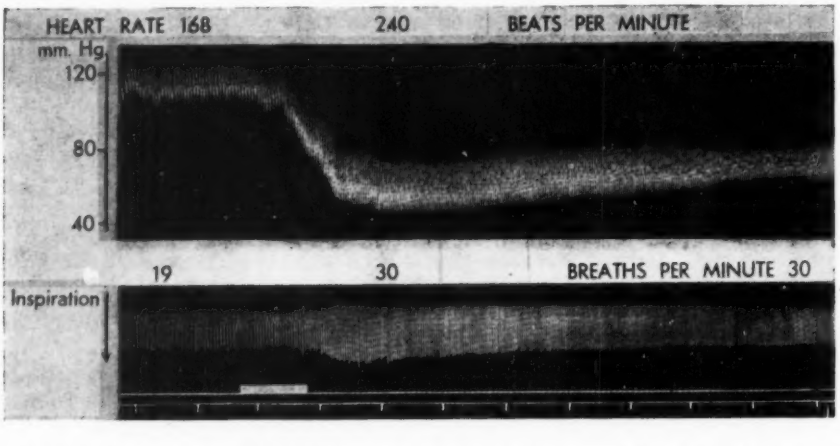


FIG. 1.—Male dog, 14 kg., pentobarbitone. At signal 1 g./kg. of nicotinamide dissolved in 30 ml. saline injected intravenously. Tracings from above downwards: carotid blood pressure; respiratory movements; signal; time in minutes.

The extended recovery period after intravenous injection of nicotinamide may be related to the time required for clearance of the drug from the circulating blood. The results of periodical determinations (Fig. 2) show an exponential decrease of the blood concentration after intravenous injection. A few minutes after injection into dogs, cats, and rabbits only about 20–30% of the total amount is recoverable from the blood. This calculation is based on the assumption that the blood volume is 7% of the total body weight. Thus when 0.5 g./kg. nicotinamide is injected intravenously, these 500 mg. = 4,170 μmoles are distributed within 70 ml. of blood, and we should find immediately after injection a concentration of 59.6 μmoles/ml. The experimental value of 13 μmoles/ml. thus represents about 22% of the calculated maximum. Hence it can be concluded that nicotinamide is distributed very rapidly throughout the extracellular fluid. However, 3–4 hours pass by before

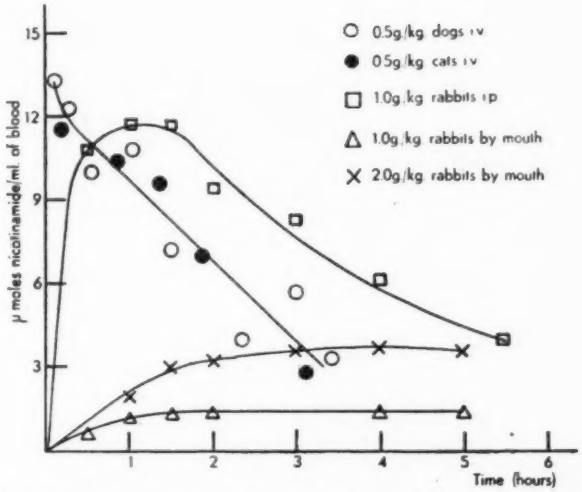


FIG. 2.—Blood concentration of nicotinamide after different routes of administration.

the blood concentration sinks below the level which can be detected by our analytical method. Nicotinamide can be removed from the blood either through the kidneys or into the intracellular space. Since the first possibility is eliminated by the experiments described below, it becomes clear that absorption of nicotinamide by the tissue cells is a rather slow process and probably dependent on an enzymatic reaction.

After intraperitoneal injection (Fig. 2), the blood concentration rises fast, and remains for about one hour at an almost constant level, while absorption into the blood stream and disappearance from the circulation proceed at the same rate. After oral administration a constant blood level of nicotinamide is maintained for several hours, since the speed of intestinal absorption regulates the passage of nicotinamide into the blood (Fig. 2).

TABLE I

THE HYPERGLYCAEMIC EFFECT OF NICOTINAMIDE ON FASTING ANIMALS

| Mean of | 2 Rabbits | 2 Rabbits | 2 Rabbits | 4 Rats | 4 Rats |
|---|---|---|---|---|---|
| Dose g./kg. | 0·75 i.v. | 1·0 i.p. | 2·0 by mouth | 1·0 i.p. | 0·75 s.c. |
| Min. After Administration | mg. Glucose/100 ml. of Blood | | | | |
| 0 | 124 | 120 | 94 | 79 | 95 |
| 5 | 128 | — | — | — | — |
| 30 | 168 | 132 | 135 | 132 | 106 |
| 60 | 203 | 153 | 138 | 139 | 121 |
| 90 | 214 | 173 | 156 | 159 | 132 |
| 120 | 209 | 195 | 229 | 177 | 148 |
| 180 | 182 | 213 | 174 | 173 | 120 |
| 240 | 148 | 204 | 174 | 131 | 101 |
| 300 | 134 | 177 | 174 | — | 96 |

*Effect on Blood Sugar.*—Nicotinamide has been shown to protect rats against alloxan diabetes (Lazarow, Liambies, and Tausch, 1950), although the mechanism of this effect is not clear. It was thus surprising to find that nicotinamide produces a large hyperglycaemia, which reaches its maximum after 1–1½ hours, but is still pronounced after 3–4 hours (Table I). After intraperitoneal injection and oral administration a similar effect is obtained both in normal and anaesthetized animals. It is noteworthy that after injection of nicotinamide histological examination of the dog's liver revealed degenerative changes with vacuolization both in the peripheral and central parts of the lobules.

*Oliguria.*—When we tried to examine the excretion of nicotinamide in the urine of our experimental animals, we observed a considerable decrease in urine flow. In Fig. 3 is shown the effect of intraperitoneal injection of nicotinamide into rats which had been given 50 ml. water/kg. by stomach tube. At a dosage of 1 g./kg. of nicotinamide, urine excretion is almost completely suppressed, while smaller quantities, 0.5 g./kg. and above, cause oliguria, though the total excretion of water finally approaches that of the controls. The effect is approximately proportional to dosage. Intraperitoneal injection of nicotinamide (1 g./kg.) is less effective when the water is also given intraperitoneally than when given by mouth. This may be due to dilution of nicotinamide. A still smaller

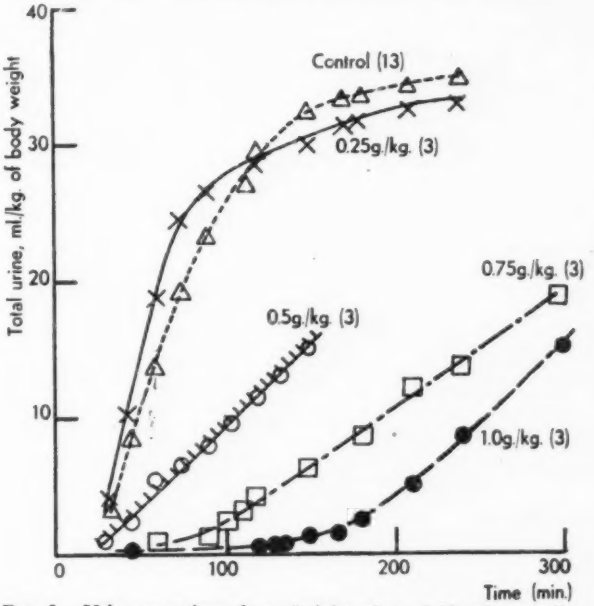


FIG. 3.—Urine excretion after administration of 50 ml. water/kg. by stomach tube and simultaneous i.p. injection of varying doses of nicotinamide. Mean figures from groups of four rats each. Number of groups in brackets.

oliguria is observed when both nicotinamide and water are given by mouth.

If 2.5% saline is given instead of water, the rate of urine excretion is increased and the total amount excreted during the experiment is larger than the saline intake. Nicotinamide reduces the rate of saline excretion, indicating that it does not act through stimulation of the posterior lobe of the hypophysis (see Discussion). In accordance with these findings, the oliguria caused by nicotinamide in hypophysectomized rats (Table II) was very great, although it proved impossible to use the same dosage as in normal animals owing to the greater toxicity of the drug in the operated rats.

TABLE II

INFLUENCE OF NICOTINAMIDE ON THE EXCRETION OF URINE IN HYPOPHYSECTOMIZED RATS

| Dose g./kg. | No. of Rats | Total Urine Excretion in ml./kg. After (Minutes) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 30 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 300 | 360 |
| 0 | 2 | 4·0 | — | 5·4 | 11·2 | — | — | — | — | 17·4 | — |
| 0 | 4 | 2·15 | — | 6·2 | — | 10·0 | — | 12·2 | — | 16·0 | — |
| 0 | 4 | 5·0 | 7·0 | 9·4 | 10·8 | 13·7 | — | 18·1 | 18·1 | — | — |
| 0·4 | 2 | — | 1·6 | 3·0 | 5·4 | — | — | — | — | 9·4 | — |
| 0·5 | 4 | — | — | 0·8 | 3·5 | 5·7 | 7·5 | 9·7 | 11·4 | — | — |
| 0·6 | 4 | — | — | — | — | — | 2·2 | — | — | 4·05 | 7·0 |

Groups of rats received 50 ml. of sterilized water/kg. of body weight intraperitoneally and varying doses of nicotinamide subcutaneously.

Finally the effect of nicotinamide on mercurial diuresis was studied. We produced a suitable diuresis in normal rats by injecting the mercurial 2–2½ hours before giving water. Nicotinamide was again very effective in producing oliguria under these conditions, as after administration of saline.

On histological examination of the kidneys the glomeruli appeared normal. The tubular epithelium was swollen and there was hydropic degeneration of the cells lining the collecting tubules. In some places the swelling had led to an obstruction of the tubular lumen; the interstitial tissue showed oedema.

TABLE III

EXCRETION OF NICOTINAMIDE (OR DERIVATIVE)

On the first day groups of four rats received 50 ml. water/kg. body wt. by stomach tube and varying doses of nicotinamide by intraperitoneal injection; water was allowed after six hours. On the second day 50 ml. water/kg. body wt. was given by stomach tube, and water was allowed after four hours. Both urine volume and nicotinamide excretion are calculated per kg. body wt.

| Dose of nicotinamide: | 0·75 g./kg. | | 1·0 g./kg. | |
|---|---|---|---|---|
| Time After Injection in Hours | Urine ml. | mg.NA | Urine ml. | mg.NA |
| 1st day 2 | 2·4 | 12·3 | — | — |
| 3 | 0·5 | 5·5 | 3·3 | 30·0 |
| 4 | 1·1 | 28·0 | 1·9 | 31·2 |
| 5 | 4·4 | 17·7 | 5·9 | 55·0 |
| 6 | 2·9 | 4·0 | 4·5 | 34·5 |
| 24 | 19·7 | 109·0 | 22·6 | 240·0 |
| 2nd day 1 | 6·3 | 13·8 | 5·1 | 15·4 |
| 2 | 22·2 | 7·9 | 18·4 | 23·6 |
| 3 | 11·7 | 1·0 | 4·1 | 1·0 |
| 4 | 4·1 | 1·0 | 0·6 | 0 |
| 24 | 16·2 | 30·6 | — | — |
| | Total (recovered) | 230·8mg. (32·5%) | | 430·7mg. (43%) |

*Fate of Nicotinamide in the Body.*—About 12 hours after administration of nicotinamide the urine excretion of rats becomes normal and remains so. During this period the urine contains up to 10 mg./ml. of nicotinamide (or a derivative of it) depending on dosage. The total amount recovered during 72 hours varies between 25 and 50%, as shown in Table III.

## Discussion

The oliguria which follows administration of nicotinamide could be due to a number of causes. The fall in blood pressure could lead to a reduction of renal blood flow and thus of glomerular filtration. There is, however, no correlation between the duration of the hypotension and of the oliguria, and the blood pressure fall was never sufficient to account for complete anuria. Moreover, if this explanation were correct, the return of the blood pressure to normal should be followed by a large urine flow (Rydin and Verney, 1938). In our experiments the rate of urine production lags considerably behind the normal for a number of hours. An indirect action of nicotinamide via the hypophysis has also been excluded. Additional support for this comes from the experiments with saline diuresis, since Brunn (1920) and Gibbs and Fulghum (1946) have shown that the antidiuretic hormone is less effective during a saline diuresis. The only factor to which urine production can be clearly related is the blood concentration of nicotinamide. When the latter falls below a certain level, urine flow starts again, although the excretion of the drug or its metabolites extends over two to three days. It thus becomes probable that nicotinamide acts directly on the kidney in proportion to its blood level by increasing tubular reabsorption. This conclusion is supported by the effect of nicotinamide on saline and mercurial diuresis and by the histological changes observed in the kidneys. It is apparent that nicotinamide is reabsorbed from the glomerular filtrate within wide limits and thus also influences the reabsorption of water. This suggests that the substance actually excreted in the urine is not nicotinamide but a metabolite (see Beyer, Russo, Gass, Wilhoyte, and Pitt, 1950).

The hyperglycaemic action of nicotinamide is another expression of the metabolic changes induced by this substance. It was first thought

that the liver cells are damaged by the high doses of nicotinamide and that the stored glycogen is then converted into glucose. However, analysis of the glycogen content of the liver did not give consistent results. Another possibility is that nicotinamide interferes with the utilization of glucose by the cells. This hypothesis is based on the inhibitory effect of nicotinamide on those enzymes which contain nicotinamide in their prosthetic group (Feigelson, Williams, and Elvehjem, 1951). Nicotinamide produces a suppression of endogenous respiration and could well be responsible for the diminished utilization of glucose. The fact that Lazarow and co-workers (1950) did not observe the hyperglycaemic effect of nicotinamide in rats, although they injected 0.9 g./kg. intravenously, is probably due to differences in experimental procedure (the first blood sample was taken by them after three hours and all samples were pooled).

## Summary

1. A method for the determination of nicotinamide in biological fluids has been developed.

2. The toxicity of nicotinamide does not vary greatly with the route of injection, but is lower after oral administration.

3. Massive doses of nicotinamide (0.5–1.0 g./kg.) injected intravenously or intraperitoneally cause a fall in blood pressure and a rise in the rate and amplitude of breathing and sometimes in the pulse rate.

4. Nicotinamide injected intravenously is distributed very rapidly throughout the extracellular space, but absorption by the tissue cells is a slow and prolonged process.

5. Nicotinamide produces a marked hyperglycaemia.

6. Nicotinamide produces oliguria preceded, after large doses, by a period of anuria. This effect is not mediated by the pituitary but appears to be due to a direct action on the tubular mechanism.

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# THE ANTIHISTAMINE AND ANTIADRENALINE PROPERTIES OF A SERIES OF N-NAPHTHYLMETHYL-2-HALO-ETHYLAMINE DERIVATIVES

BY

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Dibenamine (N: N-dibenzyl-2-chloroethylamine hydrochloride) was described by Nickerson and Goodman (1945, 1947) as a specific antagonist of the effects of injected adrenaline and of stimulation of the sympathetic nervous system. A relationship between chemical structure and pharmacological action was postulated (Nickerson, Nomaguchi, and Goodman, 1946). Later Nickerson and Gump (1949) examined some 200 analogues of dibenamine, among which were four compounds included in the series reported here.

The nature of the antagonism to adrenaline shown by these compounds, of the structural type R R′N.$CH_2CH_2Cl$, was reported to be different from that of any other known series of antiadrenaline compounds (Nickerson, 1949). The essential difference lay in the fact that the dibenamine type of compound exerted an absolute or non-competitive antagonism to adrenaline whereas compounds such as the ergot alkaloids, yohimbine, F883, or F933 exerted a competitive type of inhibition.

Loew and Micetich (1948a) showed that some of these haloalkylamine derivatives possess a strong antihistamine action in addition to their antagonism to adrenaline.

The object of this investigation was to examine quantitatively some of the properties of a closely related group of these compounds (Chapman, James, Graham, and Lewis, 1951), paying particular attention to the specific activity in inhibiting the actions of histamine, adrenaline, and adrenergic nerve stimulation.

## METHODS

*Acute Toxicity.*—The LD50 was estimated by Karber's method in male white mice weighing 20–25 g., mepyramine maleate being included with the compounds under examination (referred to by code numbers J5–J28, see Table I). Readings were taken at 1, 24, 48, and 72 hours. The aqueous solubility of some of these compounds was not sufficient for the tests; arachis oil or an aqueous solution of propylene glycol was then used as solvent (see Table I). Fine suspensions of a few substances had to be used. All injections were made in a volume of 1 ml./100 g. of mouse.

*Cats.*—Cats anaesthetized with chloralose (60 mg./kg.) were prepared for recording the carotid blood pressure; injections were made into the external jugular vein. To one group adrenaline (4 μg./kg.), histamine (2 μg./kg.), and acetylcholine HBr (1 μg./kg.) were administered before and five minutes after increasing doses of each compound, until the response to adrenaline was reversed, that to the other agents inhibited, or toxic symptoms were manifested. The haloethylamines are long-acting, and for this reason, when assessing the results of the test, the doses were considered as being cumulative.

In a further group of cats histamine (2 μg./kg.) was injected before and five minutes after each of three to five dose levels of compound. The doses selected were based on information obtained from the first group of experiments. The results were variable, and so a scatter diagram was made of the percentage inhibition of the fall of blood pressure due to histamine, plotted against the logarithm of each dose, and the regression line was calculated for each compound. The ED75 was read from these and compared with that of mepyramine.

In another group of cats anaesthetized with chloralose four doses of histamine (1, 2, 4, and 8 μg./kg.) and three doses of adrenaline (4, 8, and 16 μg./kg.) were regularly and repeatedly injected before and at intervals of 5, 30, 60, 120, 180, and 240 minutes after a suitable dose of each haloethylamine. In this way the course of the inhibition with time was followed.

A series of spinal cats was used to evaluate the effects of the compounds on the pressor response to adrenaline, according to the technique of Chen, Nash, and Russell (1950) and Chen and Russell (1950), either alternating a standard dose of adrenaline (4 μg./kg.) or graded doses of 2, 4, 8, and 16 μg./kg. of adrenaline with increasing doses of a haloethylamine. In most animals contractions of the nictitating membrane were also recorded. Similar experi-

ments, injecting graded doses of histamine and antagonist, were carried out on cats anaesthetized with chloralose.

*Dogs.*—In dogs anaesthetized with sodium pentobarbitone (40 mg./kg.) injected intraperitoneally, the pressor effect of 4 μg./kg. of adrenaline injected into the right femoral vein, and the pressor response to 30 seconds' carotid occlusion, were recorded from the left femoral artery. Increasing doses of haloethylamines were administered until the pressor response to adrenaline had been reversed and the pressor response to carotid occlusion inhibited.

*Guinea-pig Aerosol.*—Groups of 15 guinea-pigs of 300–400 g. weight were exposed to a 0.5% solution of histamine as a spray, of particle size 0.5–2.5 μ (mean size 1 μ). This was delivered into a box fitted with an exit tube leading to waste at a rate of 4 l./min. The time elapsing between onset of spray and collapse from asphyxia was recorded and the animal resuscitated in a stream of 5% $CO_2$ in $O_2$. Twenty-four hours later the same animals were injected subcutaneously with haloethylamines, one dose level to each group. Each animal was exposed to the aerosol one hour after injection, and the protective effect was assessed. After seven days the test was repeated, a different dose level for each compound being used. As animals developed tolerance to the effects of the histamine aerosol they were replaced. Mepyramine was included as a reference compound and assessment of potency made by comparing the ED75 obtained from a plot of percentage increase of time to asphyxia against logarithm of dose of compound.

*Guinea-pig Ileum.*—The mean response to at least four additions of histamine (1–3 μg./100 ml. Tyrode's solution, in a 10-ml. bath, *p*H 7.3, temperature 37° C., 5% $CO_2$ in $O_2$) was recorded and a dose of haloethylamines left in contact for five minutes before repetition. As these compounds have a prolonged action only one reading could be made from each piece of intestine. The ED75 was recorded by plotting the percentage inhibition against the logarithm of the dose, and was used for comparison with mepyramine.

Various preparations of isolated perfused rabbit and cat heart, isolated auricle, and isolated intestine and uterus of rabbit were made and the effect of varying doses of haloethylamines on the responses to adrenaline and histamine investigated.

*Enzymatic Oxidation.*—The effect of amine oxidase (from guinea-pig liver) on the oxidation of adrenaline was observed according to the method of Blaschko, Richter, and Schlossman (1937). Varying concentrations of haloethylamines ($10^{-7}$–$10^{-3}$) were added during separate trials.

The action of histaminase (serum of pregnant women in the third trimester) on histamine was examined according to the method of Kapeller-Adler (1949), and the effects of varying concentrations of the compounds observed.

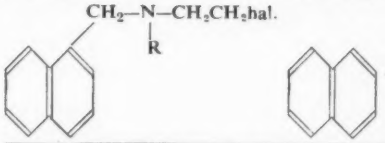
## Results

The code numbers and structure of the 19 compounds examined are listed in Table I.

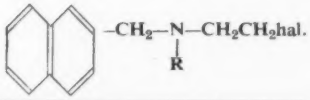
TABLE I

STRUCTURE AND ACUTE TOXICITY (I.P. MICE) OF NAPHTHYLMETHYL-2-HALOETHYLAMINE DERIVATIVES

N-1-naphthylmethyl-2-haloethylamine derivatives (J5–J19).




N-2-naphthylmethyl-2-haloethylamine derivatives (J20–J28).




| Code No. | R | Halogen | Solvent | LD50 (Karber) mg./kg. 48 hrs. | Death in |
|---|---|---|---|---|---|
| Mepyramine | | | Water | 157 | 1 hour |
| J5 | Phenyl | Cl | *Arachis oil | 2,500 | 2–3 days |
| J6 | ,, | Br | * ,, ,, | 1,000† | 2–3 ,, |
| J7 | ,, | I | * ,, ,, | 2,000 | 2–3 ,, |
| J10 | Ethyl | Cl | Water | 128 | 24 hours |
| J11 | ,, | Br | ,, | 42 | 24 ,, |
| J12 | ,, | I | ,, | 42 | 24 ,, |
| J13 | ,, | F | ‡Dil. HCl | 350 | 1 hour |
| J16 | Methyl | Cl | Water | 162 | 2 hours |
| J17 | ,, | Br | ,, | 49 | 24 ,, |
| J18 | ,, | I | ,, | 57 | 24 ,, |
| J19 | ,, | F | ,, | 200 | 2 ,, |
| J20 | Ethyl | Cl | ,, | 90 | 24 ,, |
| J21 | ,, | Br | ,, | 20 | 1–2 days |
| J22 | ,, | I | Watery soln. propylene glycol | 11 | 1–2 ,, |
| J23 | ,, | F | ‡Dil. HCl | 135 | 4 hours |
| J25 | Methyl | Cl | Watery so'n. propylene g'ycol | 138 | 1–2 days |
| J26 | ,, | Br | Water | 40 | 1–2 ,, |
| J27 | ,, | I | Watery soln. propylene glycol | 40 | 1–2 ,, |
| J28 | ,, | F | ‡Dil. HCl | 180 | 4 hours |

All Cl derivatives were used as hydrochlorides, Br derivatives as hydrobromides, I derivatives as hydriodides. J13, J23, and J28 are bases which were disso'ved in hydrochloric acid just before injection, as the salts are very hygroscopic. J19 is a hydrochloride.

* 5–10% so'ubility in arachis oil. At higher concentrations a finely divided suspension was used.

† 540 mg./kg. at 72 hours.

‡ Before injection alkali was added until c'oudiness appeared.

*Toxicity.*—The toxic symptoms were fairly constant throughout the series, being a general depression of the central nervous system followed by cardiovascular and respiratory failure. The exceptions were the fluoroethylamines (J13, J19, J23, J28) which caused prolonged convulsions, although after a few hours a terminal phase of depression or exhaustion occurred, especially with J19, J23, and J28. Some of the chloro derivatives caused excitement of short duration at first. J5, J6, and J7 were relatively innocuous, although this may be partly due to poor solubility. The halogen derivatives showed decreasing toxicity in the order I, Br, Cl, F; this agrees with the descending order of molecular weights. The LD50s are given in Table I.

It is perhaps significant that death was generally delayed for 12–24 hours, in some instances longer. Again the exceptions were the fluoro derivatives which killed within one hour (J13), in 1–2 hours (J19), or within four hours (J23, J28). Death was due to asphyxia. J5, J6, and J7 caused some delayed deaths after 2–3 days.

*Antiacetylcholine Action.*—None of the compounds affected the depressor response to acetylcholine (Fig. 1) or the pressor response to pituitrin.

*Antiadrenaline Action.*—The majority of the compounds antagonized to a greater or less degree the pressor response to adrenaline (Fig. 1C). J5, J6, and J7 had little activity, only J6 causing any significant inhibition of the pressor response. On the other hand, J10, J11, and J12 were most active, and are about as potent as any antiadrenaline agent so far reported. The fluoro derivative of this group, J13, was inactive, as also were the other fluorides. Activity decreased gradually in the other members of the series; the chloro-compounds were noticeably less active than either the bromo- or iodo-compounds.

When graded doses of the inhibitor agent were injected alternately with the standard dose of adrenaline, the pressor response to the latter was reduced in steps until it was abolished. A linear relationship was established between the probit of the percentage inhibition and the logarithm of the dose (Bliss, 1935; Chen *et al.*, 1950).

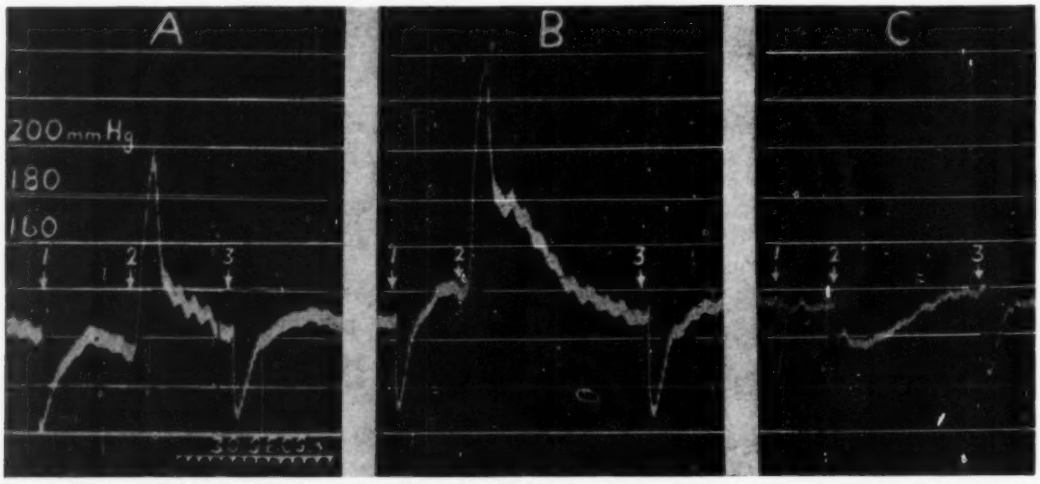



FIG. 1.—Cat, ♂, 3 kg. wt. chloralose 60 mg./kg. Carotid blood pressure. Time in 30 sec. A, Standard responses to histamine 2 μg./kg. (1), adrenaline 4 μg./kg. (2), and acetylcholine 1 μg./kg. (3). B, 5 min. after J18 100 μg./kg. C, 5 min. after J18 1.5 mg./kg. Note potentiation of response to adrenaline with the smaller dose and reversal with the larger dose, which inhibits the effect of histamine. Neither dose level affects responses to acetylcholine.

TABLE II

ADRENERGIC INHIBITION (EFFECTIVE DOSES IN MG./KG.)

| Compound | Cats | | Dogs; Sodium Pentobarbitone | |
|---|---|---|---|---|
| | Chloralose. Reversal of Pressor Response to Adrenaline | Spinal. 99% Reduction of Pressor Response to Adrenaline | Reversal of Pressor Response to to Adrenaline | Inhibition of Pressor Response to Carotid Occlusion |
| J5 | — | — | | |
| J6 | 20 | — | 20 | — |
| J7 | — | — | | |
| J10 | 0·5–1·0 | 0·17 | 0·50 | 5·50 |
| J11 | 0·5–1·0 | 0·23 | 0·20 | 3·50 |
| J12 | 0·5–1·0 | 0·57 | | |
| J13 | — | — | | |
| J16 | 8–10·0 | 21·4 | | |
| J17 | 0·5–1·0 | 0·47 | 0·25 | 2·5 |
| J18 | 1·0–1·5 | 4·17 | | |
| J19 | — | — | | |
| J20 | 5–7 | 8·90 | | |
| J21 | 1·0–2·0 | 5·30 | 1·5 | 3·50 |
| J22 | 2·0–2·5 | 4·50 | | |
| J23 | — | — | | |
| J25 | — | — | | |
| J26 | 3·0 | 6·75 | 2·5 | 3·50 |
| J27 | 3·0 | 5·37 | | |
| J28 | — | 50·0 | | |

— Indicates inactivity. Blank indicates not tested.

The dose corresponding to 99% inhibition for each compound was taken as a measure of activity and is shown in column 1 of Table II. The ED99 rather than the dose bringing about reversal was chosen because the phenomena of reversal cannot be obtained regularly in spinal cats with a low blood pressure.

When graded doses of adrenaline (2, 4, 8, and 16 μg./kg.) were used in addition to graded doses of antagonist (Fig. 2) it could be shown that before the pressor response was completely inhibited in a spinal cat (or reversed in a cat anaesthetized with chloralose) the action was of a competitive nature, the response depending upon the dose of agonist and antagonist.

Gaddum's equation for competitive antagonism $K_1C_1=[1+(K_2C_2)^n]^a/1-a$ (Gaddum, 1943) was

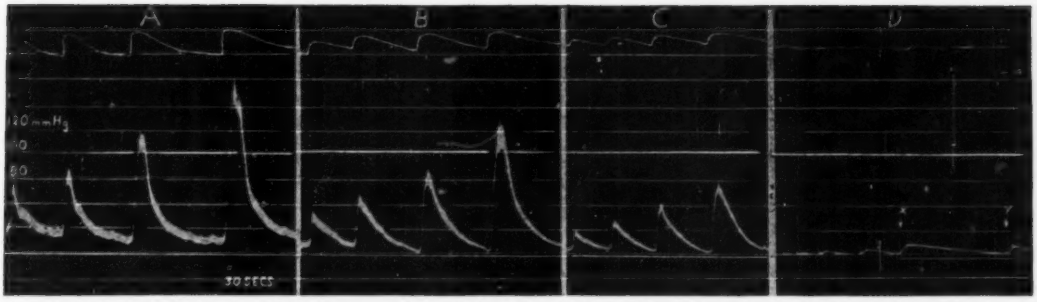



FIG. 2.—Cat, ♂, 2.8 kg. wt. Spinal preparation, nictitating membrane (above), and carotid blood pressure (below). Time in 30 sec. A, normal responses to 2, 4, 8, and 16 μg./kg. adrenaline. B, the responses to similar doses 5 min. after J17, 25 μg./kg. C, 5 min. after J17, 150 μg./kg. D, responses to the same doses of adrenaline, and also to 300 μg./kg. and 1 mg./kg. at X and Y respectively, 5 min. after J17, 300 μg./kg. Note in B and C that the inhibition is competitive in nature (the response is related to the dose of adrenaline); this is not so in D. The small residual pressor responses to the larger doses of adrenaline are probably due to cardiac stimulation which is not inhibited by the J compounds. The nictitating membrane record is similar to the blood pressure record.

employed to test the nature of this antagonism by the method described by Chen and Russell (1950). The equation was satisfied for all the active compounds. An example is shown in Fig. 3. The value of $n$ was always found, empirically, to be 2, which agrees with the observation of Chen and Russell (1950) on J11 (SY28).

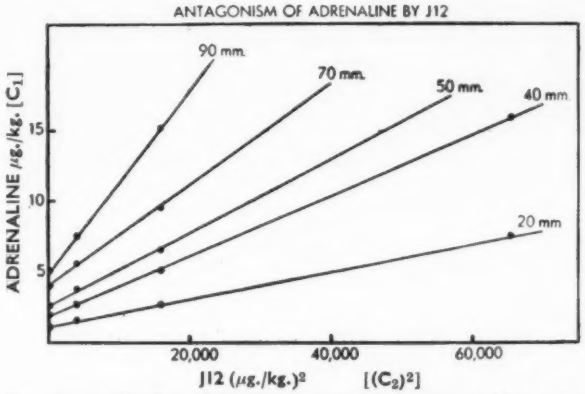



FIG. 3.—Application of Gaddum's equation for competitive antagonism $K_1C_1=[1+(K_2C_2)^n]^a/1-a$ to the effect of compound J12 in small doses (50–275 μg./kg.) on the pressor responses to adrenaline in a spinal cat. The abscissa is the square of the dose of antagonist in μg./kg., the ordinate is the amount of adrenaline injected in μg./kg. The pressor responses to several doses of adrenaline were measured and equipressor amounts of adrenaline determined after injecting various dose-levels of J12. The linearity of the relationship indicates the competitive nature of the antagonism exerted by J12 in these doses against adrenaline.

Once completely inhibited by larger doses of antagonist the dose of adrenaline can be increased to 1 mg./kg. or more and no pressor response is evident (Fig. 2). At this stage the equilibrium between adrenaline and antagonist had disappeared.

The action of the haloethylamines investigated is like that of other haloalkylamine derivatives in that they antagonize the excitatory actions of adrenaline, e.g. on cat pregnant uterus, rabbit pregnant and non-pregnant uterus, and cat nictitating membrane, but do not affect the inhibitory actions of adrenaline, e.g. on cat non-pregnant uterus, rabbit intestine, or the effect of adrenaline on the heart. It was observed that in high concentrations (250–500 μg./100 ml.) the haloethylamines depressed the tone and movement of isolated rabbit intestine and that this direct action masked the response to adrenaline.

In small doses (10 μg.) some of these compounds stimulated isolated perfused rabbit hearts. These doses inhibited the stimulant action of histamine, but not that of adrenaline, nor the inhibitory action of acetylcholine. Larger doses of these compounds (250 μg. to 1 mg.) inhibited cardiac action. Similar effects were found with the isolated auricle. The tone of the non-pregnant cat uterus *in situ* was lowered by large doses of the haloethylamines (20–30 mg./kg.) so that it no longer responded to adrenaline.

When injected *in small doses* (20–60 μg./kg.) the haloethylamines potentiated the pressor response to adrenaline (Fig. 1B). This effect was produced even by the most potent adrenaline inhibitors, J10, J11, and J12, as well as those compounds which did not antagonize adrenaline at all, such as J13 and J25. In the potent antiadrenaline compounds, however, this effect was not very great. With J13 and J25, the potentiation was considerable, reaching 100%. It is not due to atropine-like activity, occurring in spinal cats and atropinized cats.

*Adrenergic Inhibition.*—Stone and Loew (1948) and others showed that haloalkylamine derivatives (e.g. SY28—J11 in our series) not only reversed the pressor effects of adrenaline but also inhibited responses due to sympathetic nerve stimulation.

We have found, in agreement with others, that there was a considerable difference in the dose required to reverse the response to adrenaline and

that necessary to inhibit the pressor response to carotid occlusion. These doses for some of our haloethylamines are given in columns 3 and 4 of Table II.

The blood pressure is frequently lowered by the haloethylamines, usually more so in the cat than in the dog. It is likely that this effect is at least partly due to inhibition of the sympathetic tone in the peripheral vascular bed.

TABLE III
ANTIHISTAMINE ACTIVITY

| Compound | Blood Pressure Cats (Chloralose) ED75 (mg./kg.) | Guinea-pig Aerosol ED75 (mg./kg.) | Guinea-pig Ileum ED75 (μg./100 ml.) |
|---|---|---|---|
| Mepyramine maleate .. | 0·43 | 0·09 | 0·27 |
| J5 | >25·0 | 9·34 | >100·0 |
| J6 | >25·0 | 9·00 | >100·0 |
| J7 | >25·0 | 8·50 | >100·0 |
| J10 | 0·26 | 0·08 | 0·40 |
| J11 | 0·29 | 0·10 | 0·69 |
| J12 | 0·31 | 0·10 | 0·62 |
| J13 | >25·0 | >25·0 | >100·0 |
| J16 | >25·0 | 0·20 | 0·89 |
| J17 | 1·72 | 0·11 | 0·83 |
| J18 | 1·34 | 0·14 | 1·20 |
| J19 | >25·0 | >25·0 | 80·00 |
| J20 | 6·76 | 3·55 | 5·31 |
| J21 | 8·51 | 1·45 | 3·63 |
| J22 | 8·13 | 1·86 | 4·79 |
| J23 | >25·0 | 20·0 | >100·0 |
| J25 | >25·0 | 4·36 | 17·00 |
| J26 | 9·55 | 2·10 | 13·80 |
| J27 | 16·20 | 2·40 | 15·85 |
| J28 | 20·0 | 25·0 | 90·0 |

*Antihistamine Action.*—Antihistamine action was strongest in J10, J11, and J12. The fluoro-derivatives, J13, J19, J23, and J28, were inactive. The other members of the series were less active than the former group, and it was noticeable that the chloro-derivatives were less active than the bromo- or iodo-derivatives. The ED75 for each compound for three different methods of assessment of potency is shown in Table III. They are in good agreement except for the fact that the two chloro-derivatives J16 and J25 are inactive against the fall of blood pressure caused by histamine, whereas they are active against the effects of histamine on the isolated ileum or when inhaled by guinea-pigs. In dogs the depressor response to histamine was not reduced by more than 50–60% by any of the compounds. Mepyramine was used as a standard of comparison throughout the experiments, and it will be seen that J10, J11, and J12 are as active as this substance, if not more so. Application of Gaddum's equation demonstrated the competitive nature of the antagonism to the effects of histamine on the blood pressure of cats.

*Time-Action Relationship.*—The effects of both histamine and adrenaline are inhibited within five minutes of intravenous injection of a haloethylamine (Figs. 4 and 5), even if the solubility is poor as with J27. The results of the previous tests show that the two properties of the compounds (antiadrenaline and antihistamine) when present run parallel throughout this small series—e.g., J10 inhibits both agents powerfully, whereas J13 inhibits neither.

However, this latter test revealed some difference between the nature of the inhibition of adrenaline and that of histamine (Graham and Lewis, 1951). The responses to all doses of adrenaline were reversed so that there was 100% reduction in the pressor responses to adrenaline, and absolute or non-competitive antagonism was established within a few minutes. This was not so with histamine, as, although all responses were reduced, they were not abolished. The response was dependent upon the doses of antagonist and of histamine, showing that the antagonism was of a competitive nature (Figs. 4 and 5).

The time of action is another factor separating these two properties. The onset of action was the same, but the durations were different. The antagonism to histamine began to wear off quickly, whereas it was several hours before the effects of the antagonism to adrenaline began to disappear. In about 1½ hours the responses to histamine were almost normal, but it was four hours before the absolute antagonism of the pressor responses to adrenaline became competitive in nature.

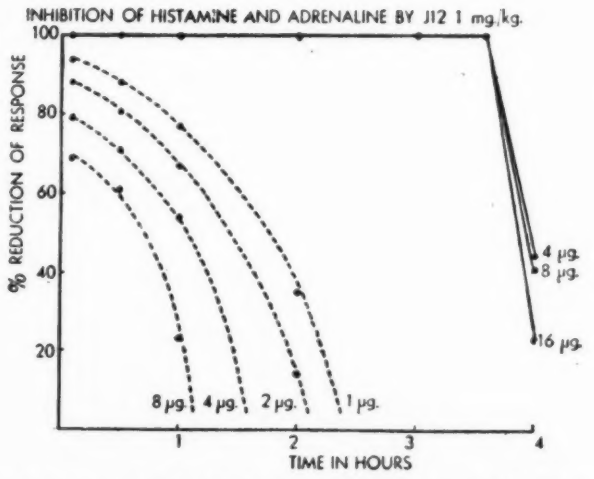



FIG. 4.—The relationship of antiadrenaline and antihistamine action in J12. Ordinate, percentage reduction of the response; abscissa, time in hours. At zero time 1 mg./kg. of J12 was injected i.v. into a cat under chloralose. Graded doses of adrenaline and histamine were injected before and at fixed intervals after J12. Reversal of the response to adrenaline is recorded as 100% reduction. Adrenaline (uninterrupted lines) was inhibited in a "non-competitive" manner for 3½ hours. Histamine (interrupted lines) was inhibited in a "competitive" manner, as shown by the fact that the response depended upon the dose of histamine.

It was observed that a dose of inhibitor producing competitive antagonism did not progress with time to produce an absolute antagonism. These remarks apply to all the compounds examined.

*Actions on Enzymes.*—One phase of the action of haloethylamines on the pressor response to adrenaline was potentiation. Several of the compounds were tested for their effect upon the enzymic oxidation of adrenaline. It was observed that they antagonized the action of amine oxidase to about the same degree—i.e., 20–80% within the range of concentration $10^{-4}$–$10^{-3}$. This holds whether the compounds are potent antiadrenaline agents or not.

The action of histaminase on histamine was studied in only a small number of experiments, as the purpose was to confirm that the antihistamine action of the haloethylamines was not due to potentiation of the enzyme. On the contrary, slight inhibition was observed. The action was not studied quantitatively.

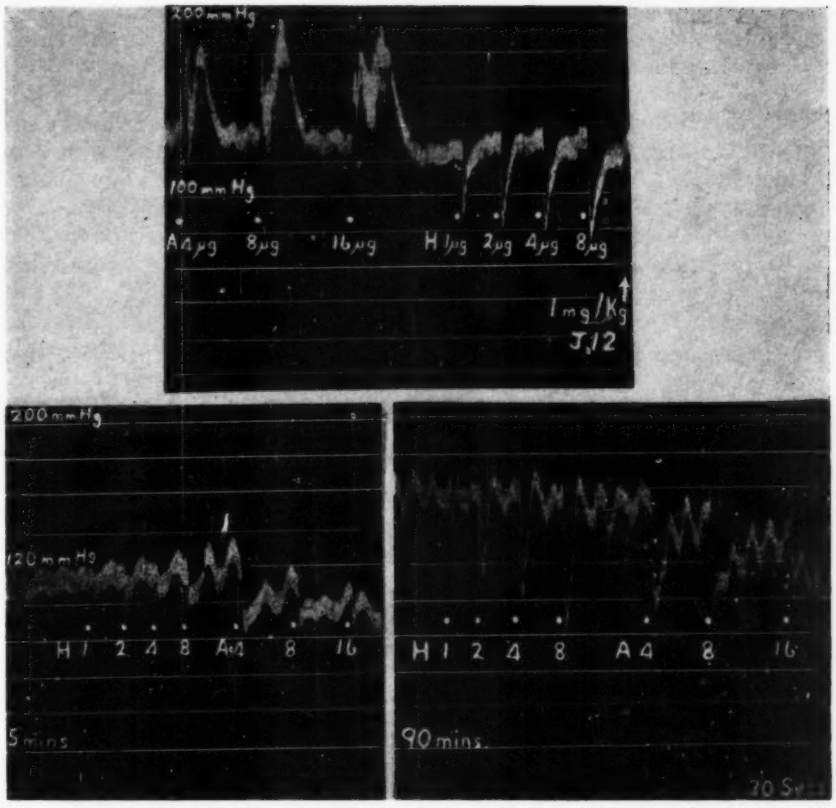



FIG. 5.—Kymograph record illustrating Fig. 4. Cat, ♂, 2.7 kg./wt. Chloralose 60 mg./kg. carotid blood pressure, time in 30 sec. Injections of adrenaline (4, 8, 16 µg./kg.) and histamine (1, 2, 4, 8 µg./kg.) before and at 5 and 90 min. interval after J12, 1 mg./kg. i.v.

## DISCUSSION

The structural features in this series of 2-haloethylamines which favour antagonism to adrenaline also favour antagonism to histamine. Parallellism between these two actions is a constant feature of the results, increasing antiadrenaline potency going with increasing antihistamine potency, and decline in one activity accompanying diminution of the other. Thus substitution in the 1-position on the naphthyl ring and the presence of an ethyl group on the N-atom favour activity, whereas the 2-naphthylmethyl moiety and the presence of phenyl or methyl groups on the N-atom produce a less active molecule. Loew and Micetich (1948a) also found a loss of activity with alteration from the 1- to the 2-position in the naphthyl ring in the mouse protection test, and Nickerson and Gump (1949) confirmed this on cat blood pressure. The halogen in the haloethyl side-chain also alters the activity (Chapman *et al.*, 1951), fluorine in the side-chain rendering the molecule inactive. The bromides and iodides having a 2-naphthyl ring and/or a methyl group show much greater activity than do the corresponding chlorides. Thus we do not regard the methyl and 2-naphthyl moieties as having such an inactivating influence as Nickerson and Gump (1949) and Loew and Micetich (1948a) did. These authors only examined the chloro-derivatives in this series. Variation in halogen is as important as other variations in the molecular structure.

Nickerson and Gump (1949) have suggested that the halogen in the side-chain of the 2-haloalkylamines splits off and the resulting carbonium ion undergoes cyclization to form a pharmacodynamically active cyclic immonium ion. Fluorine being chemically immobile, it might be supposed that cyclization would not occur in fluoro-compounds, which would be inactive. Inactivity as antiadrenaline and antihistamine agents has been found in the fluoro-compounds in this series and a marked difference in acute toxicity from the other haloalkylamines, which supports the theory of Nickerson and Gump (1949) concerning the mode of

formation of active intermediates. A noticeable feature is the relative weakness of activity in two chloro-compounds, viz., N-methyl-N-1-naphthylmethyl-2-chloroethylamine (J16) and N-methyl-N-2-naphthylmethyl-2-chloroethylamine (J25) in contrast to the considerable activity in the bromo- and iodo-analogues. Investigations at present being undertaken into the reactivity and kinetics of cyclization *in vitro* of these compounds may explain this anomaly.

One of the most potent antiadrenaline agents in the series is J11 (N-ethyl-N-1-naphthylmethyl-2-bromoethylamine HBr), also known as SY28 (Loew and Micetich, 1948b). Several reports as to the actions of this compound have appeared.

A dose of haloethylamine which is inadequate to cause inhibition usually causes potentiation, some compounds being more potent in this respect than others. Such an effect passes off with time and does not alter its nature to inhibition. A dose which is enough to cause an inhibition of the response to adrenaline may display one of two alleged types of action. The first of these is the competitive type of inhibition where the degree of the pressor response depends on the relative amounts of 2-haloethylamine and adrenaline injected. The second type as defined by Nickerson (1949) occurs when, " once an adequate block has been established, it cannot be overcome by massive adrenergic stimulation " ; thus when larger doses of the 2-haloethylamines are given no pressor responses are elicited by 10 mg./kg. of adrenaline.

The use of the pressor reflex following upon occlusion of the carotid arteries in dogs is a recognized technique for eliciting activity in the sympathetic nervous system (Heymans and Regniers, 1929). Agents which are known to inhibit the pressor response to injected adrenaline may be assumed to diminish reflex responses by peripheral action rather than by ganglionic block or inhibition of the afferent side of the reflex (Stone and Loew, 1948). In this respect the most potent compound is J17, N-methyl-N-1-naphthylmethyl-bromoethylamine HBr. The administration of 0.5 mg./kg. of J17 causes a fall in blood pressure in cats and dogs of some 40 mm. Hg and four hours' duration.

As stated, there is a general correlation between molecular structures favouring antiadrenaline activity and those favouring antihistamine activity. There are, however, certain differences between these two activities (Graham and Lewis, 1951), viz., the shorter duration of the antihistamine action of any effective dose of 2-haloethylamine ; the absence of a dose range causing potentiation of responses to histamine ; the fact that the antihistamine activity is always within the competitive range even with large doses of powerful inhibiting compounds which exhibit an extreme degree of " non-equilibrium " inhibition of adrenaline ; and the inhibition of the action of histamine but not of adrenaline on the heart. Nickerson and Harris (1949) in a short note suggest that antihistamine activity in a large series of 2-haloethylamines develops in two stages with time—firstly a competitive inhibition and then a non-competitive inhibition. The test object used was guinea-pig ileum, and no details of doses, duration of effect, etc., are available. Our results with 2-haloethylamines (0.1–1.0 $\mu$g.) in doses sufficient to abolish the response of isolated guinea-pig ileum to added histamine (0.1–0.3 $\mu$g.) do not support this. Such adequate doses left in contact with the tissue for some hours retain a competitive type of inhibition, larger amounts of histamine (10 $\mu$g.) causing a contraction. The studies on blood pressure reported confirm the competitive nature of the inhibition of histamine, in the doses used. Larger doses of these compounds " permanently " lower the blood pressure, which modifies responses to histamine. Even in toxic doses, the responses to large doses of histamine have never been abolished completely.

The antihistamine potency of some of these compounds is very high, being equal to that of mepyramine. The indices of potency compared with mepyramine differ according to the preparation and technique employed for the assay. This is usual, but the overall picture is very consistent.

The inhibitor effect on preparations of amine oxidase is of interest in relation to the potentiating action of comparable concentrations on the pressor response to adrenaline. Other agents, such as mepyramine, which potentiate adrenaline in certain doses (Graham, 1949) also inhibit amine oxidase (Tickner, 1951).

This action of our haloethylamines is not due to the reactive intermediate immonium ion, as the antihistamine and antiadrenaline properties appear to be. This is shown by the fact that compounds which do not form immonium ions, e.g., fluorides (Chapman *et al.*, unpublished), exert an equivalent degree of inhibition on the enzyme. None of the other structural rearrangements made in this series affect the inhibitory action on amine oxidase.

The antihistamine effect is obviously not due to potentiation of the activity of histaminase any more than is that of mepyramine (Kapeller-Adler, 1951). Whether or not it is due to formation of the same cyclic immonium intermediate product as effects antiadrenaline activity is not finally settled, but seems probable.

While not as potent as the phenoxy-haloethylamines (Nickerson and Nomaguchi, 1951) this series of 2-haloethylamines contains some of the most specific and most powerful antiadrenaline and antihistamine agents known, with both properties in the one molecule.

## Summary

1. We have examined a series of N-naphthylmethyl-N-(aryl or alkyl)-2-haloethylamines and reported on their antiadrenaline and antihistamine properties.

2. The structural configurations which favour antiadrenaline action also favour antihistamine action. The presence of 1-naphthylmethyl in the molecule is favourable, while the 2-naphthylmethyl moiety produces a less active molecule. When the aryl or alkyl group is ethyl, activity is usually greater than when it is methyl, while activity is minimal when it is phenyl.

3. The importance of the nature of the halogen atom in the haloethyl side-chain is stressed. Bromine is usually most favourable, whereas fluorine produces an inactive molecule.

4. These compounds do not appreciably affect the responses to acetylcholine.

5. These compounds in small doses potentiate the pressor response to adrenaline; the activity of amine oxidase is also inhibited.

6. Inhibition of adrenaline is competitive in low dosage but becomes non-competitive when a larger dose is used.

7. Inhibition of histamine is always of a competitive nature.

8. The action of histamine on the heart of rabbits is inhibited by active compounds; that of adrenaline is not.

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# MECHANISM OF THE PREVENTION OF NICOTINE CONVULSIONS BY HEXAMETHONIUM AND BY ADRENALINE BLOCKING AGENTS

BY

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In a previous paper (Laurence and Stacey, 1952a) we have shown that hexamethonium and pentamethonium protect mice and rats against nicotine convulsions to a marked degree, but that they afford no protection against the action of a number of other convulsants nor against insulin convulsions (Laurence and Stacey, 1952b). In the present work we have sought an explanation for this protection.

Hexamethonium does not pass freely into the cerebrospinal fluid (Paton), so that there are good reasons for investigating possible peripheral mechanisms. Evidence has already been advanced for believing that, although violent stimulation of the carotid body may, according to Schmidt and Comroe (1940), lead to convulsions, it is not by blocking afferent impulses from this structure that hexamethonium exerts its protection (Laurence and Stacey, 1952a). Attention was therefore turned to other peripheral actions of nicotine which are modified by hexamethonium. Hexamethonium blocks stimulation of the adrenal medulla by nicotine, and it was thought possible that the adrenaline liberated might play a part in precipitating nicotine convulsions, since adrenaline is known to facilitate spinal reflexes (Bülbring and Burn, 1941) and the passage of impulses across synapses (Bülbring and Burn, 1942), and to potentiate the action of various substances on the central nervous system (Friedemann and Elkeles, 1932). The convulsant action of nicotine was therefore examined under conditions in which the effects of sympathetic stimulation were increased (by simultaneous administration of adrenaline or noradrenaline) or decreased (by blocking agents or adrenalectomy).

## METHODS

Mice and rats were used. In each experiment a comparison was made between the convulsion rates in two groups of animals of the same sex, from the same colony, and of similar weights and ages which had received the same dose of a convulsant (either nicotine or leptazol). One group had received previously an injection of a solution of the drug whose anticonvulsant action was being examined, while the other had received an equal volume of solvent. For dibenamine the time interval between the two injections was four hours, for all other drugs 30 minutes. *Mice* were given nicotine (0.2 mg. base/ml.) or leptazol (2 mg./ml.) into a tail vein, the injection occupying exactly 10 seconds timed by a metronome. Premedication was by intraperitoneal injection. *Rats* received nicotine (2 mg. base/ml.) subcutaneously or leptazol (25 mg./ml.) intraperitoneally and premedication by subcutaneous injection. Each experiment was repeated after one week with the groups crossed over; the results were then summed. Doses of convulsants were adjusted to give a suitable convulsion rate for the purpose of the experiment. In *mice* these were: nicotine 0.2–0.4 mg./kg., leptazol 20–40 mg./kg.; and for *rats*: nicotine 2–3 mg./kg., leptazol 50–70 mg./kg. In the experiments in which adrenaline, noradrenaline, and pitressin were used, these substances were added to the solution of the convulsant and used at once.

Adrenalectomized animals had all recovered completely from the operation before use and were not receiving cortical steroids.

Probability figures were obtained from Mainland's fourfold contingency tables (Mainland, 1948).

## RESULTS

### *Effect of Premedication with Various Substances on the Convulsion Rate in Normal and Adrenalectomized Animals*

It will be seen from Table I that all adrenaline blocking agents tested protected against nicotine convulsions but not against leptazol convulsions; tolazoline had the weakest action. Hexamethonium, artane, and diparcol also afforded protection, hexamethonium being much the most potent. All the other drugs tested (atropine,

hyoscine, ergometrine, and promethazine) gave no protection in doses up to 15 mg./kg.

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON THE NICOTINE CONVULSION RATE. CORRESPONDING FIGURES FOR THE LEPTAZOL CONVULSION RATE ARE GIVEN IN BRACKETS

| "Antagonist" | Dose mg./kg. | Animals | No. of Animals Convulsed out of 20 | | P |
|---|---|---|---|---|---|
| | | | Control | Exptl. | |
| Dibenamine HCl | 5 | Rats | 19 (*13*) | 2 (*15*) | <0·01 |
| ,, ,, | 5 | Mice | 15 | 5 | |
| Yohimbine HCl | 5 | Rats | 16 (*14*) | 0 (*16*) | |
| ,, ,, | 5 | Mice | 18 | 10 | |
| SKF 688-A .. | 5 | Rats | 14 | 1 | |
| Ergotamine tartrate | 5 | ,, | 16 | 0 | |
| DHE methane sulphon. .. | 5 | ,, | 9 | 0 | |
| ,, ,, | 5 | Mice | 18 | 13 | 0·06 |
| Tolazoline HCl | 15 | Rats | 15 | 1 | <0·01 |
| ,, ,, | 5 | ,, | 16 | 16 | — |
| Hexamethonium $I_2$ | 5 | Mice | 17 | 3 | <0·01 |
| ,, ,, | 2 | Rats | 15 (*17*) | 4 (*16*) | |
| Artane ,, .. .. | 15 | ,, | 12 | 6 | 0·06 |
| ,, .. .. | 5 | ,, | 16 | 14 | — |
| Diparcol .. .. | 15 | ,, | 14 | 5 | <0·01 |
| ,, .. .. | 5 | ,, | 6 | 7 | — |
| TEAB .. .. | 40 | ,, | 11 | 8 | 0·26 |
| Atropine sulphate | 5 | ,, | 10 | 9 | — |
| ,, ,, .. | 15 | ,, | 5 | 8 | — |
| Hyoscine HB .. | 15 | ,, | 8 | 13 | — |
| Ergometrine maleate .. | 5 | ,, | 10 | 10 | — |
| ,, | 15 | Mice | 11 | 17 | — |
| Promethazine HCl | 15 | Rats | 12 | 9 | 0·26 |

When similar experiments were performed using adrenalectomized rats (Table II) some protection was still afforded by hexamethonium and dibenamine; the figures suggest that this is less than with intact rats, while tolazoline now gave no significant protection. It was also noticed that adrenalectomized rats were slightly less sensitive to nicotine than intact rats.

TABLE II

EFFECT OF ADRENALINE BLOCKING AGENTS ON THE NICOTINE CONVULSION RATE IN ADRENALECTOMIZED RATS

| "Antagonist" | Dose mg./kg. | No. of Rats Convulsed out of 20 | | P |
|---|---|---|---|---|
| | | Control | Exptl. | |
| Hexamethonium $I_2$ | 2 | 14 | 7 | <0·05 |
| Dibenamine HCl .. | 5 | 16 | 10 | |
| Tolazoline HCl .. | 15 | 11 | 9 | — |
| ,, ,, .. | 5 | 11 | 12 | — |

### *Effect of Adrenaline and Noradrenaline on the Convulsant Action of Nicotine and Leptazol*

The effect of mixing adrenaline and noradrenaline with the convulsants was next investigated; the results are recorded in Table III. Experiments 1–4 show that both substances potentiated the action of both nicotine and leptazol. A number of experiments were performed, of which examples only are recorded here, with the intention of bringing to light any difference between the actions of adrenaline and noradrenaline in this respect, but no constant differences could be established.

TABLE III

EFFECT OF ADRENALINE, NORADRENALINE, AND PITRESSIN ON NICOTINE AND LEPTAZOL CONVULSION RATES

| Expt. | Convulsant | "Adjuvant" | No. of Mice Convulsed out of 20 | | P |
|---|---|---|---|---|---|
| | | | Control | Exptl. | |
| 1 | Nicotine | Adrenaline 40$\gamma$/kg. | 3 | 9 | 0·05 |
| | ,, | ,, 80$\gamma$/kg. | | 12 | 0·01 |
| 2 | ,, | ,, 40$\gamma$/kg. | 2 | 10 | <0·01 |
| | ,, | Noradrenal. 40$\gamma$/kg. | | 9 | |
| 3 | Leptazol | Adrenaline 40$\gamma$/kg. | 2 | 14 | <0·01 |
| | ,, | Noradrenal. 40$\gamma$/kg. | | 14 | |
| 4 | ,, | Adrenaline 20$\gamma$/kg. | 1 | 9 | <0·01 |
| | ,, | Noradrenal. 20$\gamma$/kg. | | 6 | <0·05 |
| 5 | Nicotine | Pitressin 0·4 i.u./kg | 3/10 | 3/10 | — |
| | | ,, 1·2 ,, ,, | | 2/10 | — |
| | | ,, 4·0 ,, ,, | | 2/10 | — |
| 6 | Leptazol | ,, 2 ,, ,, | 3 | 10 | 0·02 |
| 7 | Nicotine preceded by $C_6I_2$ 5 mg./kg. i.p. | ,, 2 ,, ,, | 5 | 13 | 0·01 |

### *Effect of Pitressin on the Convulsant Action of Nicotine and Leptazol*

The results recorded above suggested that the effect of other pressor substances might be of interest. Pitressin was selected, as there is no evidence that it has any central action. When it was added to leptazol, potentiation was observed (Table III, Experiment 6), but over a wide range of dose no potentiation of nicotine was found (Experiment 5). If, however, the circulatory disturbances provoked by nicotine were inhibited by a previous injection of hexamethonium to both groups of rats, the potentiation of nicotine by pitressin became evident.

## DISCUSSION

The first series of experiments shows that two groups of drugs among those investigated antagonize nicotine convulsions, namely, ganglion-blocking substances and adrenaline-blocking substances. This antagonism is well marked with hexamethonium with doses as low as 2 mg./kg., but is also easily demonstrable with artane and diparcol when doses of 15 mg./kg. are given. This dose level of diparcol is required to produce

ganglion blockade (Heymans, Estable, and Castillo de Bonneveaux, 1949). If the protection against nicotine convulsions afforded by these substances depends on their ganglion blocking action, tetraethylammonium might be expected to afford protection also. According to Paton and Zaimis (1949) it has a blocking action on sympathetic ganglia only 1/20th that of hexamethonium. This may account for the absence of definite anticonvulsant action at the maximum tolerated dose of 40 mg./kg. Of the adrenaline blocking substances tolazoline provided least protection, 15 mg./kg. being required to give protection comparable with that given by 5 mg./kg. of the other compounds. This is in accordance with its "weaker adrenergic blocking action" found by Nickerson and Smith (1949). These experiments accord with the view that sympathetic block, whether this takes place at ganglia or at adrenergic nerve endings, protects against nicotine convulsions.

When adrenalectomized animals were used, protection was still afforded by hexamethonium and dibenamine, though there was evidence that this was less complete, and tolazoline failed to protect even at the higher dose. The effects of sympathetic stimulation by nicotine in these animals would be less than in intact animals, and sympathetic block might therefore be expected to have less action.

The results of the experiments with adrenaline and noradrenaline, which agree with those of Friedemann and Elkeles (1932), who showed that adrenaline and pituitrin potentiate the action of a number of narcotics and convulsants, can be interpreted as indicating that increasing the effects of sympathetic stimulation increases the convulsant action of nicotine and leptazol.

From these experiments it is concluded that the convulsant action of nicotine is increased by the sympathetic stimulation it causes, and that substances reported here as protecting against nicotine convulsions do so by diminishing those effects of nicotine caused by sympathetic stimulation. Other convulsants, such as leptazol, in whose action sympathetic stimulation does not play an important part, are not antagonized.

When we come to consider the way in which sympathetic stimulation could potentiate the action of a convulsant, two main possibilities present themselves : (i) changes in the circulation might lead to a higher concentration of the convulsant being reached in cerebral tissue, either by an increased cerebral blood flow or by a raised blood pressure resulting in a more rapid passage of the convulsant through the blood-brain barrier, or (ii) adrenaline and noradrenaline might facilitate the development of convulsions by a direct action on the central nervous system.

Though our experiments do not give a final answer as to the relative importance of these two factors, some further deductions may be drawn. We have found that pitressin will potentiate the action of leptazol and, in certain circumstances, of nicotine, and since there is no evidence that pitressin has any direct action on the brain it presumably potentiates by a vascular mechanism. Vascular changes caused by adrenaline and noradrenaline might therefore well cause a potentiation of the order observed. This is confirmed by the observation of Friedemann and Elkeles (1932) that adrenaline and pituitrin increase the passage into the brain of those substances which normally pass the blood-brain barrier. Nicotine, adrenaline, noradrenaline, and pitressin all cause a violent rise in blood pressure, but their effect on cerebral flow in the doses used is not known. King, Sokoloff, and Weschler (1952) have shown that in man adrenaline increases while noradrenaline decreases cerebral flow, but the doses they used were much smaller than ours, and, apart from species differences, no conclusion could be safely drawn from them as to the changes in cerebral flow in our experiments.

It may be concluded that a rise in blood pressure, possibly accompanied by an increased cerebral blood flow, is probably an important factor in the potentiation of convulsants by excitation of sympathetic effector organs.

Considering now the second of the two possibilities mentioned above, our experiments have not shown what part, if any, a central action of adrenaline or noradrenaline plays in the development of nicotine convulsions. This point is being investigated further. Where the central effects of adrenaline and noradrenaline have been compared, as in their effect on the tremor of Parkinsonism (Barcroft, Peterson, and Schwab, 1952), and on the oxygen consumption of the brain (King *et al.*, 1952), adrenaline has been found to produce marked changes and noradrenaline none. In our experiments adrenaline and noradrenaline appeared to have equal effect, but our doses were so much greater that a difference in central action might have been obscured.

Finally, our experiments show that the circulatory disturbances caused by a drug may markedly modify its action on the central nervous system, so that in assessing the potency of a drug acting on the central nervous system the part played by

accompanying changes in the cerebral circulation must be assessed before any theoretical conclusions can be drawn.


## Summary

1. Hexamethonium, dibenamine, SKF 688-A, yohimbine, ergotamine, and dihydroergotamine protect mice and rats against nicotine convulsions. Tolazoline, diparcol, and artane have a similar but weaker action. These substances do not protect against leptazol convulsions.

2. Adrenalectomized rats are less effectively protected than intact rats. The simultaneous administration of adrenaline and noradrenaline potentiates the convulsant action of nicotine and leptazol. Pitressin potentiates the action of leptazol; it increases that of nicotine only if hexamethonium has first been given.

3. It is concluded that the action of nicotine on the brain is intensified by the sympathetic discharge it causes. Blocking the sympathetic system reduces its convulsant action.

4. The mechanism whereby sympathetic discharge intensifies the action of a convulsant is discussed, and it is concluded that the vascular changes produced are of major importance. Hence, in comparing the potency of drugs acting on the central nervous system it is essential that the condition of the cerebral circulation should be comparable.


We are indebted to Mr. French, of Messrs. Smith, Kline & French, for the specimen of SKF 688-A.

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# COLORIMETRIC AND BIOLOGICAL ESTIMATION OF ADRENALINE AND NORADRENALINE IN SUPRARENALS OF GUINEA-PIGS

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Adrenaline and noradrenaline can be separately estimated in a mixture either by colorimetric or biological methods. None of the methods is strictly specific, since catechol amines other than adrenaline and noradrenaline, if present, may affect the estimation. It can be shown by paper chromatography, however, whether such other catechol amines, for example hydroxytyramine, are present in any appreciable amount. Only exceptionally does this occur in suprarenal extracts (Goodall, 1951).

The differential colorimetric method described by Euler and Hamberg (1949), based on the formation of iodochromes of adrenaline and noradrenaline, is simple and reasonably accurate when applied to suitably prepared extracts. When adrenaline is present in the mixture in amounts less than 10% of the total catechol content, biological methods give more accurate estimates; whereas small amounts of noradrenaline, less than 10%, will usually be determined more accurately by the colorimetric method.

Recently Shepherd and West (1951) have reported in this journal that the colorimetric method of Euler and Hamberg yields results which differ greatly from those obtained with a biological method (blood pressure and nictitating membrane of the spinal cat). They conclude that the colorimetric method gives erroneous results.

In order to check the basis for this statement, for which we have had no previous evidence, we have estimated colorimetrically and biologically the catechol amines in preparations of guinea-pigs' suprarenals.

## METHODS

Ten preparations of suprarenals (I–X) were made from groups of 5–7 guinea-pigs weighing 300–400 g. each. The animals were killed by a blow on the neck. The right and left suprarenals were prepared separately, weighed, ground with sand, and extracted for about one hour. Preparations were made with acidulated ethanol (I, VII, and IX), with 5% trichloracetic acid (T.C.A.) (II), and with 10% T.C.A. (III, IV, V, VI, VIII and X). In this way the influence of the extraction method was tested. The volumes of extraction fluids were 20–30 ml.

The ethanol was removed by evaporation *in vacuo* and the lipids extracted with ether. The T.C.A. was removed by extraction 4–5 times with 3–4 volumes of ether. In the second group both preparations (III and IV) became pink during the extraction procedure with the ether, probably owing to some impurity in the ether. Another brand of ether was used for the subsequent preparations which yielded completely colourless extracts. The colorimetric estimations were made according to Euler and Hamberg (1949).

For the biological estimations the cat's blood pressure and the fowl's rectal caecum were used. The extracts were assayed against standards and the results computed according to Euler (1949).

In preparations VII–X the biological activity was estimated on the cat's blood pressure and expressed as adrenaline equivalents. In the Table are also given for comparison the adrenaline equivalents calculated from the colorimetric figures for adrenaline and noradrenaline and the activity ratio found biologically for adrenaline and noradrenaline, as illustrated by the following arbitrary example:

Adrenaline equivalents on the cat's blood pressure:
720 $\mu$g./g.
Activity ratio adr./noradr. on the cat's blood pressure:
0.25
Colorimetric adrenaline figure:
510 $\mu$g./g.
Colorimetric noradrenaline figure:
44 $\mu$g./g.
Calculated adrenaline equivalents:

$$510+44.\frac{1}{0.25}=686\ \mu\text{g./g.}$$

## RESULTS

The following conclusions may be drawn from the Table.

1. The figures obtained by the colorimetric method are in good agreement with those found by biological estimation. The greatest difference

TABLE
ESTIMATIONS OF ADRENALINE AND NORADRENALINE IN SUPRARENALS

| Group | Preparations | Extraction | Weight of suprarenals mg. | Colorimetric estimation μg./g. | | | Biological estimation μg./g. | | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Adr. | Noradr. | % Adr. | Adr. | Noradr. | % Adr. | |
| 1 (7 animals, 2.36 kg.) | I | Acid ethanol | 487 | 460 | 27 | 95 | 450 | + | ~90 | |
| | II | 5% trichloracetic acid (T.C.A.) | 445 | 530 | 98 | 84 | 550 | + | ~90 | |
| 2 (6 animals, 2.13 kg.) | III | 10% T.C.A. | 431 | 250 | 40 | 86 | 250 | 30 | 89 | Pink coloured extracts |
| | IV | 10% T.C.A. | 434 | 87 | 76 | 53 | 60 | 40 | 60 | |
| 3 (5 animals, 1.97 kg.) | V | 10% T.C.A. | 442 | 490 | 71 | 87 | 480 | 80 | 86 | |
| | VI | 10% T.C.A. | 438 | 550 | 80 | 87 | 500 | 70 | 87 | |
| 4 (5 animals, 2.20 kg.) | VII | Acid ethanol | 535 | 450 | 62 | 88 | 560 found (540 calcul.) | | Adrenaline equivalents μg./g. on cat's B.P. | |
| | VIII | 10% T.C.A. | 521 | 450 | 44 | 91 | 560 found (510 calcul.) | | | |
| 5 (5 animals, 2.06 kg.) | IX | Acid ethanol | 320 | 770 | 44 | 95 | 800 found (830 calcul.) | | | White animals |
| | X | 10% T.C.A. | 326 | 790 | 120 | 87 | 900 found (960 calcul.) | | | |

seen between the results using the two methods for adrenaline estimation in 8 preparations (I, II, V–X) was less than 15%, when exception is made for the deteriorating extract IV.

2. The total catechol figures were consistently much higher per mg. gland in our material than in the material used by Shepherd and West (Fig. 1).

3. There was no consistent difference between the figures, whether acid ethanol or trichloracetic acid was used for the extraction.

4. Noradrenaline was demonstrated colorimetrically in all extracts. Its presence was also observed in those extracts which were tested biologically.

5. The amount of adrenaline in the gland extracts varied between 84 and 95% of the total catechol amines in the present material.

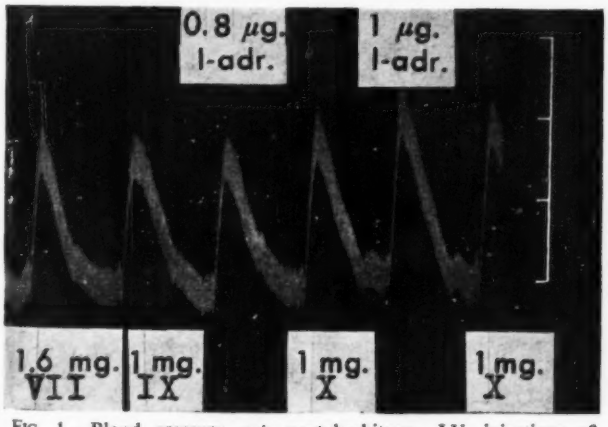


FIG. 1.—Blood pressure, cat, pentobarbitone. I.V. injections of 1.6 mg. VII and 1 mg. IX and X, compared with 0.8 μg. and 1 μg. adrenaline hydrochloride as indicated. Ordinate: 140–200 mm. Hg.

6. The estimate of biological activity obtained by assay on the cat's blood pressure, and expressed in adrenaline equivalents, showed good agreement with that calculated from the colorimetric estimation of adrenaline and noradrenaline (preparations VII–X).

7. Faulty extraction technique accounts for the low total catechol figures and high noradrenaline percentage in preparations III and IV, due to selective inactivation of adrenaline.

Paper chromatographic analysis of the extracts made with ethanol showed the presence of noradrenaline in addition to adrenaline in amounts which were of the same order of magnitude as those found biologically and colorimetrically.

When trichloracetic acid extracts were used, a displacement of the noradrenaline and adrenaline spots was observed, both showing higher $R_F$ values than normally. For this reason extraction with alcohol should be used for paper chromatographic experiments with extracts not subjected to further purification. A displacement of a similar kind has been noted previously after adsorption on alumina from trichloracetic acid extracts (Euler, 1950).

## DISCUSSION

Considerable experience of the colorimetric and biological estimations of adrenaline and noradrenaline has been had in this laboratory, yet no indication of consistent discrepancies between the two methods has been found. The results of Shepherd and West differ from ours in three respects, in (1) the total amount of catechol amines; (2) the percentage of noradrenaline; and (3) the difference between colorimetric and biological estimations.

The total amount of catechol amines might differ according to the source of the guinea-pigs; and the possibility cannot be excluded that the guinea-pigs used by Shepherd and West had a much smaller amount of suprarenal medullary catechols than ours. As regards the noradrenaline percentage, this also seems to differ in different groups of animals. Thus one of us (B. H.) found low percentages (less than 5%) of noradrenaline in most of his groups of animals. The reason for these differences is still obscure. However, the good agreement between the colorimetric and biological findings indicate that the stated differences are real.

No support has been obtained for the statement of Shepherd and West to the effect that the colorimetric method of Euler and Hamberg yields erroneous results. We can give no explanation for their observations, but it should be pointed out that low total catechol content and high percentages of noradrenaline may well occur in deteriorating extracts, as illustrated by preparations III and IV in the Table. If the biological testing is done later than the colorimetric it might then easily yield lower figures.

As to the method of biological estimation, Shepherd and West give figures in some experiments of 0 and 2% for the noradrenaline fraction of the total catechols. This surprising accuracy is rather higher than the methods which we have used would permit. In the biological as well as the colorimetric methods the accuracy usually diminishes sharply as the noradrenaline percentage approaches 10% of the total catechols. However, in some cat blood pressure preparations, even 5–10% noradrenaline mixed with adrenaline may be clearly distinguished from adrenaline alone by the different response pattern.

## Summary

The adrenaline and noradrenaline content of guinea-pigs' suprarenals was estimated colorimetrically by the method of Euler and Hamberg, and biologically by assay on the cat's blood pressure and the hen's rectal caecum with extracts made up with acid ethanol or trichloracetic acid. The catechol amine content was generally about 500 μg. per g., 5–15% being noradrenaline.

The presence of noradrenaline in the extracts was confirmed by paper chromatography.

The colorimetric method of Euler and Hamberg gave results which tallied closely with those determined biologically.

Extraction with acid ethanol or trichloracetic acid caused no consistent differences in the catechol amine figures.

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# THE EFFECT OF DENERVATION ON THE ACTION OF SYMPATHOMIMETIC AMINES ON THE NICTITATING MEMBRANE

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The work of Cannon and Rosenblueth and their collaborators on the hypersensitivity of denervated structures has given rise to a general impression that after the denervation of a tissue there is always an increased response to a chemical stimulus. However, there are notable exceptions to this rule even among the limited class of sympathomimetic amines acting on structures innervated by postganglionic sympathetic fibres. Burn and Tainter (1931) observed that the iris of the cat's eye was insensitive to the dilator action of tyramine and much less sensitive to that of ephedrine when the superior cervical ganglion was removed one or two weeks previously. On the iris of the rabbit Drake, John, Renshaw, and Thienes (1939) found that *p*-hydroxyamphetamine, which caused dilatation of the normal iris, failed to do so after denervation. Burn (1932) found that the constrictor action of tyramine and of ephedrine in the vessels of the cat's foreleg was diminished or abolished by removal of the stellate ganglion 2–3 weeks previously. These results suggested that the effect of tyramine was depressed by denervation in all tissues, but Bacq (1937) stated that this conclusion did not hold good for the nictitating membrane in the cat under Dial anaesthesia. Bülbring and Burn (1938) therefore investigated the action of tyramine in a series of cats and found that the most usual result was that the denervated membrane was more sensitive to low doses, but the normal membrane was more sensitive to high doses.

At the same time they investigated hydroxytyramine and neosynephrine (Sympatol). Their general conclusion was that the more the structure of the compound departed from that of adrenaline the more often the denervated membrane was found to respond less than the normal membrane.

While it was thus clear that substances such as tyramine and ephedrine did not stimulate denervated tissue in general as much as normal tissue, the change in the response of the nictitating membrane in the early period after the operation was still unknown. We have therefore studied this question in some detail with a wider range of substances, and have been able to show that sympathomimetic amines fall into three groups so far as their action on the denervated membrane is concerned.

## METHOD

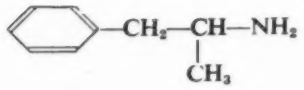
The right superior cervical ganglion was removed in an aseptic operation on each of 60 cats. After a varying interval each cat was anaesthetized with ether, and the spinal cord was divided by Dale's method (Burn, Finney, and Goodwin, 1950) without clamping the carotid arteries during the operation. The anaesthetic was discontinued and artificial respiration was given. The left cervical sympathetic chain was cut. The cat's head was then fixed rigidly and threads were passed through the nictitating membranes so that their contractions could be recorded by an isotonic lever which magnified the contractions 7.4 times. The blood pressure was recorded from one femoral artery and injections were made into the femoral vein of the opposite side.

## RESULTS

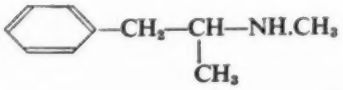
The sympathomimetic amines which were tested were found to be divisible into three groups according to their effects. The first group and the second group contained substances having only one -OH group in the ring or none ; the first group differed from the second group in that the second group contained substances having an -OH on the C atom next to the ring. The third group contained substances having two -OH groups in the ring ; it also contained the substance *m*-Sympatol or neosynephrine.

*Compounds of the First Group.*—The compounds of the first group which were examined were the following.

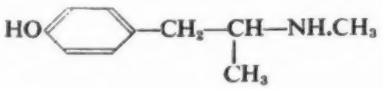
Amphetamine



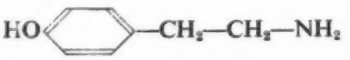
N-Methylamphetamine



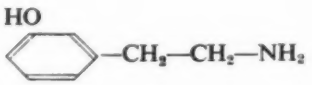
*p*-Hydroxy-N-methyl-amphetamine



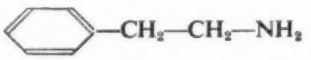
Tyramine



*meta*-Tyramine



β-Phenylethylamine



The action of the first three compounds in this group differed in one important respect from the action of the last three compounds. Each of the first three compounds possessed a $-CH_3$ group attached to the $\alpha$-carbon atom, a grouping which prevents the destruction of these substances by amine oxidase. Their action was prolonged, and a succeeding dose was given when the previous dose had produced its maximum effect; each effect was measured from the original base line.

*Early Effect.*—The effect of denervation on the response of the membrane to substances of the first group changed according to the time which elapsed after removal of the ganglion. When observations were made on the first day after operation, that is, within 19–24 hours, the denervated membrane responded much more than the normal membrane, whatever the dose injected (Fig. 1). This effect was observed in 2 cats with N-methylamphetamine, in 1 cat with *p*-hydroxy-N-methylamphetamine, in 2 cats with tyramine, and in 1 cat with β-phenylethylamine.

On the second day after operation, from 30–40 hours, the response of the denervated membrane fell below the response of the normal membrane for all but low doses. This is illustrated in Fig. 2. This change was observed in 2 cats with N-methylamphetamine and in 1 cat with β-phenylethylamine.

*Late Effect.*—When substances of this first group were examined three days after removal of the ganglion or later, the response of the denervated membrane was as a rule found to be much less than that of the normal membrane as is shown for N-methylamphetamine in Fig. 2; this substance was tested in five cats between 4 and 48 days after operation, and the result was essentially the same in all of them. Similarly, when amphetamine was tested, the results on 4 cats examined 5–7 days after denervation were the same as those with N-methylamphetamine. There was only a very slight contraction of the denervated membrane which did not vary as the dose increased, while the contraction in the normal membrane grew in proportion to the dose. The mean response in the 4 cats to 1.6 mg. amphetamine was a contraction of 40 mm. in the normal membrane, and of 5 mm. in the denervated membrane.

The third substance of this group was *p*-hydroxy-N-methylamphetamine. In 5 cats examined 3–48 days after operation the response of the denervated membrane was much less than that of the normal membrane for all but low doses (see Fig. 3). The mean response in 5 cats to the low dose of 0.2 mg. was 6 mm. in the denervated membrane, and it was 1 mm. in the normal membrane. The response

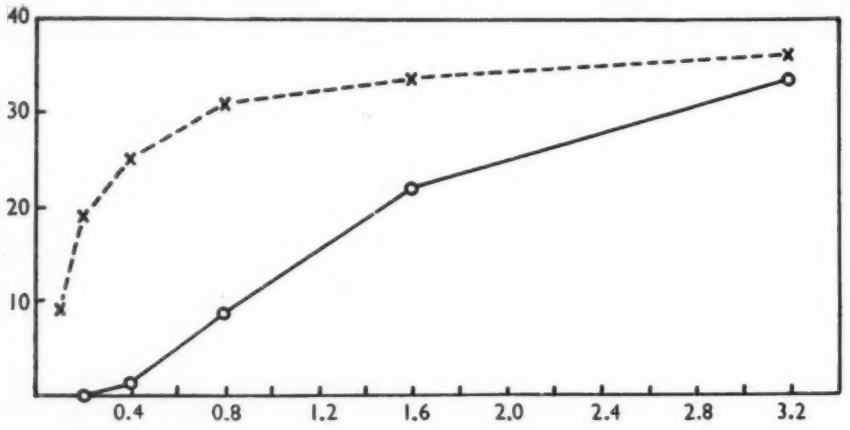



FIG. 1.—Response of nictitating membranes to N-methylamphetamine within 19–24 hours after unilateral excision of the superior cervical ganglion. Broken line is the response of the denervated membrane; full line is that of the normal membrane. Ordinates: height of contraction (mm., magnification × 7.4); abscissae: dose in mg. (mean of 2 cats).

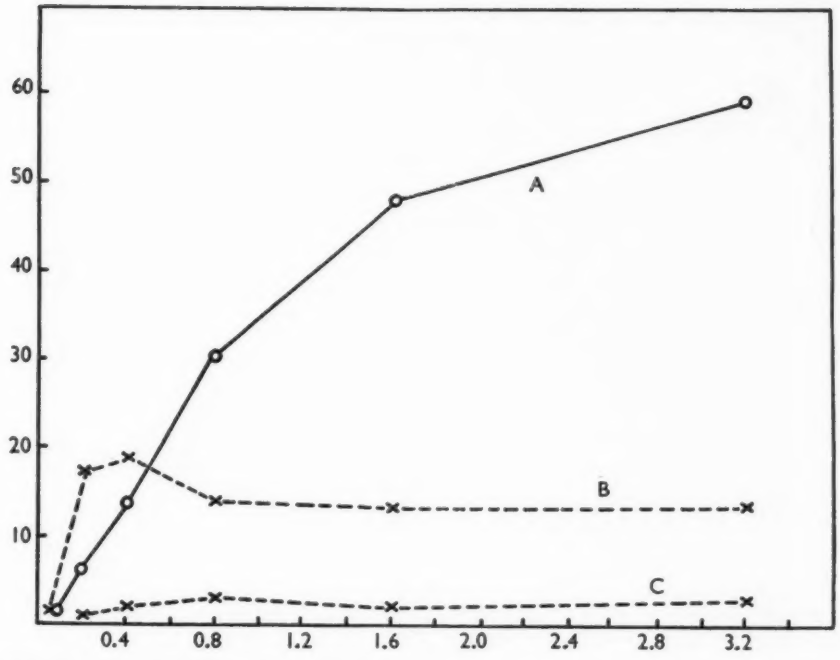



FIG. 2.—Response of nictitating membranes to N-methylamphetamine. *B* is the mean response of the denervated membrane in 2 cats, 30–40 hr. after unilateral excision of the superior cervical ganglion. *C* is the mean response in 5 cats, 4–48 days after excision of the ganglion. *A* is the response of the normal membrane in the 7 cats.

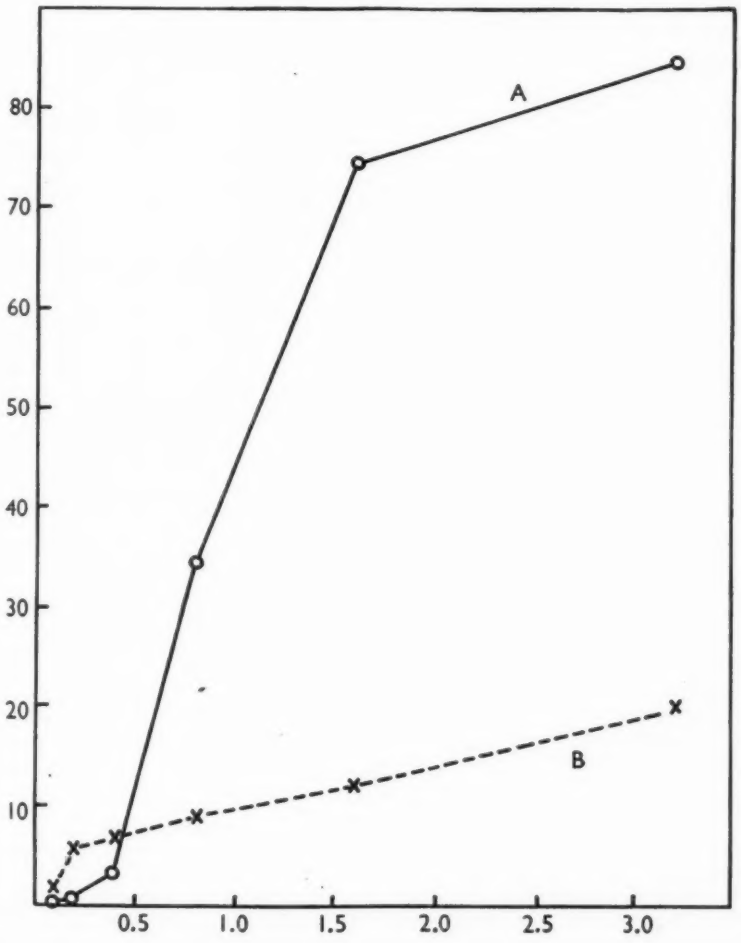



FIG. 3.—Mean response of nictitating membranes in 5 cats to *p*-hydroxy-N-methylamphetamine (pholedrine), from 3–48 days after unilateral excision of the superior cervical ganglion. *A*, normal membrane; *B*, denervated membrane. Note that the introduction of the *p*-hydroxy-group results in more effect on the denervated membrane than that seen in Fig. 2 *C*, and resembles that in Fig. 2 *B*.

of the denervated membrane to *p*-hydroxy-N-methylamphetamine when larger doses were used, though much smaller than that in the normal membrane, was not so trivial as the response to amphetamine or to N-methylamphetamine, as a comparison of Fig. 2 and Fig. 3 shows. From the results so far described with substances of the first group, the effect of removing the ganglion was to increase the response of the membrane on the side of operation to all doses during the first 24 hours, then to diminish the response when high doses were used, and finally to diminish the response to all doses. This succession of changes was not uniform for all members of the group. With amphetamine and N-methylamphetamine the succession of changes was most complete; the *p*-hydroxy derivative of the latter compound, however, when injected in low doses persisted in causing more contraction in the denervated membrane. Thus a remnant of hypersensitivity survived to this substance in the denervated membrane.

Results with tyramine obtained from observations on 15 cats examined 5–48 days after operation suggested that there was a similar succession of changes in the response of the denervated membrane, but that the rate of change varied greatly. In 1 out of the 15 cats, the response of the membrane denervated for 7 days was greater than that of the normal membrane to all doses; in 10 of the cats the response of the denervated membrane was greater than that of the normal membrane to small

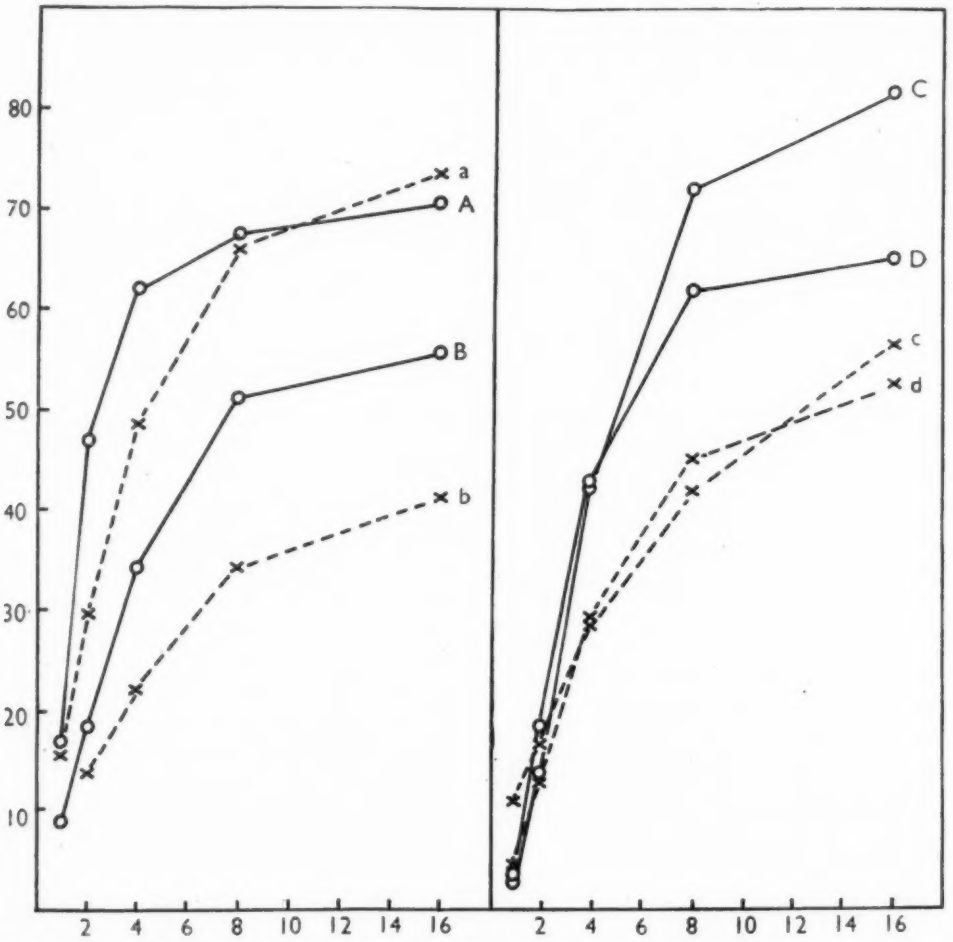



FIG. 4.—Results with substances having an —OH group on the carbon atom of the side chain next to the ring. *A* shows mean response of normal nictitating membrane to ephedrine in 6 cats; *a* shows corresponding response of membrane denervated 4–48 days. *B* shows mean response of normal membrane to β-phenylethanolamine in 4 cats; *b* shows response of membrane denervated 3–41 days. *C* shows response to *p*-hydroxy-β-phenylethano'amine in 5 cats; *c* shows response of membrane denervated 4–8 days. *D* shows response to *p*-hydroxyephedrine in 4 cats; *d* shows response of membrane denervated 4–8 days. Note that all these compounds have more action on the denervated membrane than those of the first group.

doses, but it was much smaller than that of the normal membrane to larger doses ; in the remaining 4 cats the response of the denervated membrane was always less than that of the normal membrane. These results are very similar to those described by Bülbring and Burn (1938). They also made observations on 15 cats, and instead of the number of cats in the above groups being 1, 10, and 4, they observed 2, 11, and 2 respectively.

Observations were made with *meta*-tyramine on one cat. The results were similar to those with tyramine ; the denervated membrane responded more to injections of 0.4 and 0.8 mg., but it responded less than the normal to injections of 1.6 and 3.2 mg.

Thus it appeared that the increased sensitiveness seen immediately after operation to all substances of this group could persist to tyramine perhaps indefinitely in a small proportion of cats (3 out of 30). However, in the majority of cats a change occurred in the denervated membrane within a few days after operation as a result of which there was no increase in the response of the denervated membrane with increase of dose, and the hypersensitivity was evident only for low doses.

The last substance in the group to be examined was $\beta$-phenylethylamine, which was tested in 5 cats from 3–44 days after operation. Here also the effect on the normal membrane was much greater than the effect on the denervated membrane. However, in 4 out of the 5 cats there was evidence that in response to the lowest doses the denervated nictitating membrane contracted more than the normal nictitating membrane.

Tyramine and $\beta$-phenylethylamine differed from amphetamine and N-methylamphetamine in the response of the normal membrane ; its response

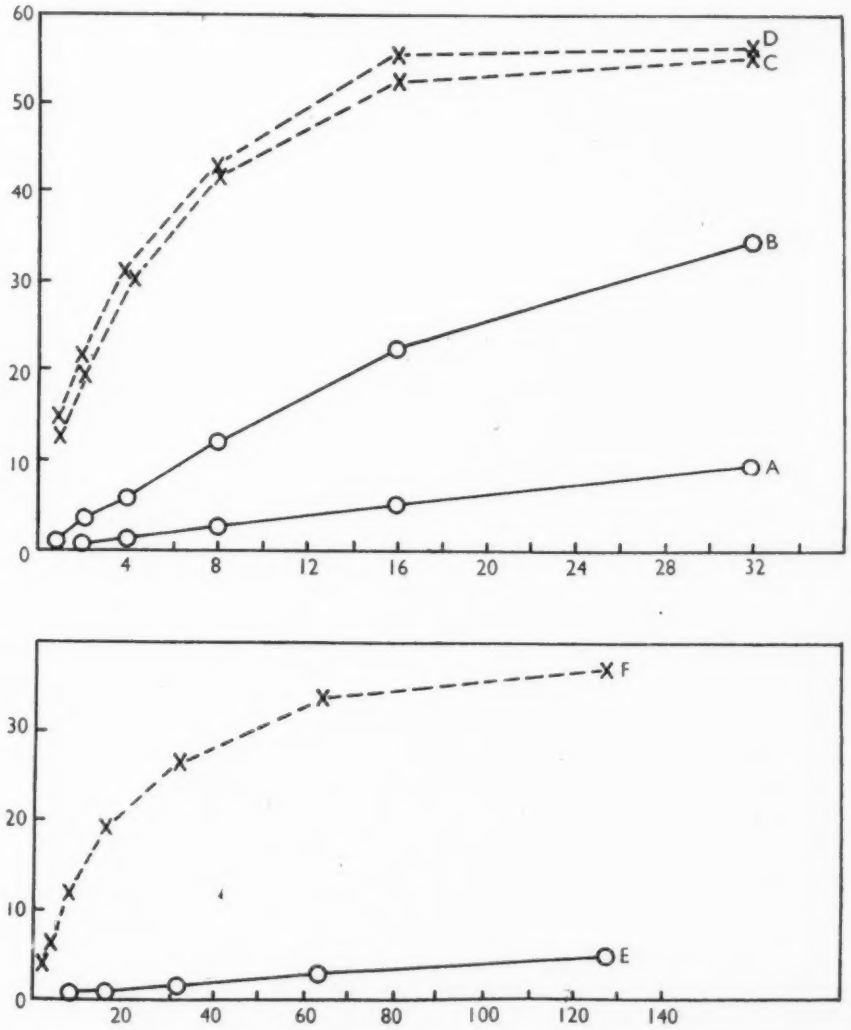
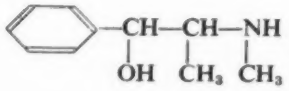



FIG. 5.—The upper chart shows the mean responses of nictitating membranes in 25 cats to noradrenaline and adrenaline. Superior cervical ganglion excised from 4–13 days. *A* is response of normal membrane to noradrenaline, *B* to adrenaline. *C* is response of denervated membrane to noradrenaline and *D* to adrenaline. The lower chart shows the mean responses in 6 cats to corbasil; ganglion excised 2–43 days. *E* is the normal response, *F* the response of the denervated membrane.

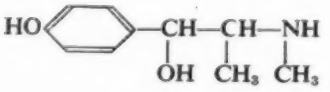
to the former substances was almost linear in relation to the dose.

*Compounds of the Second Group.*—The substances in the second group were substances containing an -OH group attached to the C atom next to the ring. They were:

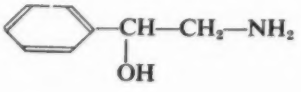
Ephedrine



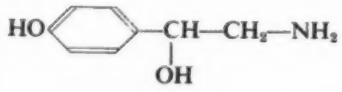
*p*-Hydroxyephedrine



β-Phenylethanolamine



*p*-Hydroxy-β-phenylethanolamine



The results obtained with these substances were similar to those of the first group in that these compounds were all active in causing contraction of the normal nictitating membrane. β-Phenylethanolamine and its *p*-hydroxy derivative both acted for a longer time than β-phenylethylamine and tyramine respectively. Again it was observed that the compounds with a $-CH_3$ group on the α-carbon atom exerted a much more prolonged effect than those without this substituent. The early effect of denervation within the first 24-hour period was to augment the response. This was observed in 4 cats with each of the substances in this group.

The later effect of the denervation was to diminish the response, though not to diminish it greatly. The mean results for all four compounds are shown in Fig. 4. *D* and *d* are the results of observations in 4 cats unilaterally denervated 4–8 days when *p*-hydroxyephedrine was injected. *C* and *c* are the similar results obtained in 5 cats denervated 4–8 days when *p*-hydroxy-β-phenylethanolamine was injected. *B* and *b* are results obtained in 4 cats denervated 3–41 days when β-phenylethanolamine was injected. With ephedrine the results were the same in 4 cats out of 6. In the other two examined respectively 7 and 9 days after operation, the denervated membrane still responded more than the normal membrane, the difference being greater when the higher doses were used. The mean results of all six cats, shown in *A* and *a* of Fig. 4, therefore indicate less difference between the response between the normal and denervated membranes than that for the other three compounds.

Since the denervated membrane continued to respond well, though less than the normal membrane, to substances of this second group for many weeks after denervation, it

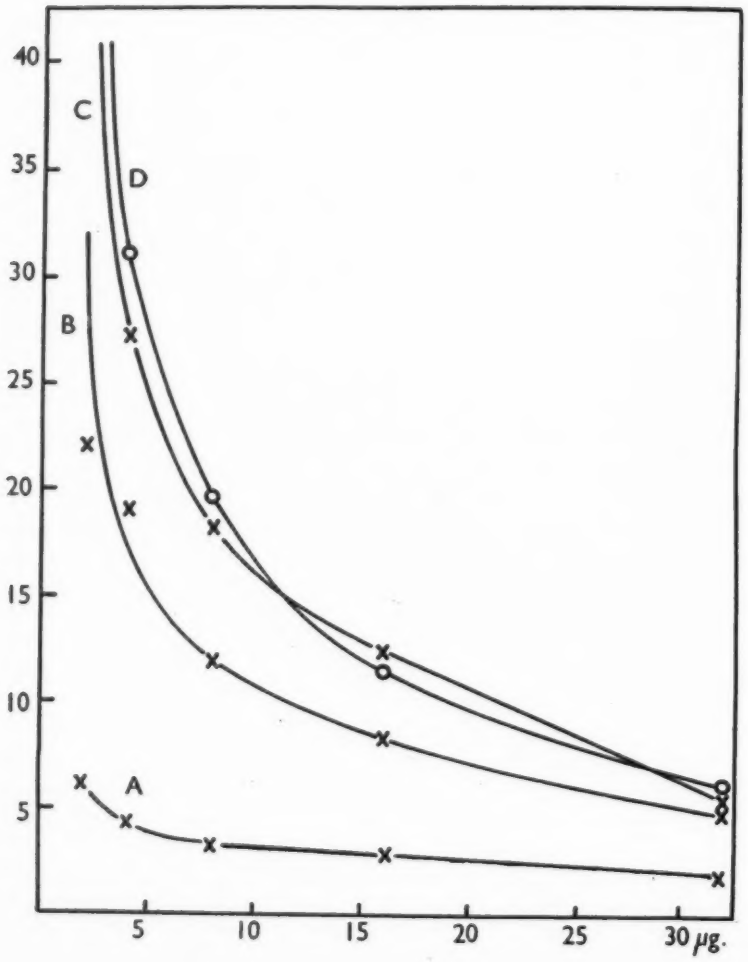



FIG. 6.—Ratios of contractions of denervated to normal nictitating membranes when noradrenaline is injected. Ordinate is ratio, abscissa is dose in μg. *A* mean result in 8 cats, 19–27 hr. denervated. *B* mean result in 6 cats, 30–66 hr. denervated. *C* mean result in 25 cats, 4–13 days denervated. *D* mean result in 8 cats, 37–50 days denervated.

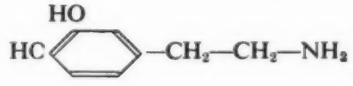
was evident that the presence of the -OH on the C atom next to the ring greatly increased the power to stimulate the denervated structure.

*Compounds of the Third Group.*—The compounds of the third group were, with one exception, compounds with two hydroxy groups in the ring in positions 3 and 4. The exception was neosynephrine, which is a substance having the same side chain as adrenaline attached to an *m*-hydroxyphenyl group. The compounds were:

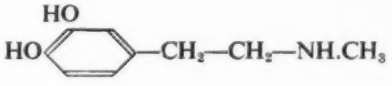
Noradrenaline

Adrenaline

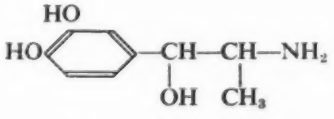
Hydroxytyramine



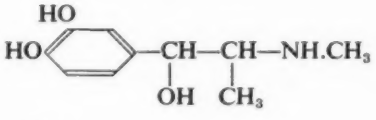
Epinine



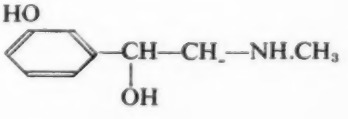
Corbasil



Dihydroxyephedrine



Neosynephrine



The effect of denervation on the response to noradrenaline and to adrenaline is already well known. The denervated membrane becomes more sensitive and remains more sensitive indefinitely, though in a small proportion of cats Bülbring and Burn (1938) found a diminished sensitivity to large doses of adrenaline.

The effect of noradrenaline on the normal membrane is very small, and less than that of adrenaline. Nevertheless, the increase in sensitivity after removal of the ganglion was found to develop much more slowly for noradrenaline than it did for the substances in the two groups already considered. While the sensitivity of the denervated membrane was greatest at 19–24 hours for the substances in the first and second groups, the sensitivity for noradrenaline at this time was not much greater than that of the normal membrane, and on the average it was not maximal until somewhere between the third and fourth day. The increase in the sensitivity of the denervated membrane to adrenaline proceeded at a faster rate than that to noradrenaline, but by the fourth day the average sensitivity of the denervated membrane was the same for both substances (Fig. 5).

The increase in the sensitivity to noradrenaline after denervation is shown in Fig. 6, in which the ratio of the contraction in the denervated membrane to the contraction in the normal membrane (the D : N ratio) is plotted against the dose. The different curves represent the mean result at various times after operation. The mean curve for cats examined from the 4th–13th day was found to be practically the same as that for cats from the 37th–50th day.

Our observations indicated that the rate of increase of sensitivity for noradrenaline was similar to that for corbasil, epinine, and dihydroxyephedrine. The similarity between corbasil and noradrenaline is evident from Fig. 5. When the D : N ratio was calculated for each of these substances and was plotted against the logarithm of the dose expressed in moles, curves approximately parallel were obtained for the four substances, as shown in Fig. 7.

Neosynephrine (*m*-Sympatol) was found to affect the nictitating membranes in the same way as adrenaline.

The similarity between the relative responses of the nictitating membranes to noradrenaline and to corbasil is of interest because the latter substance possesses a $-CH_3$ group on the $\alpha$-carbon atom whereas the former does not. Corbasil is not destroyed by amine oxidase as is noradrenaline, and indeed recent observations in this laboratory by Miss J. A. Robinson have shown that the presence of corbasil does not inhibit the action of amine oxidase on either *iso*amylamine, tyramine, or adrenaline.

The action of corbasil and of dihydroxyephedrine is also peculiar, because, although both these substances contain a $-CH_3$ group attached to the $\alpha$-carbon atom, their effect on the nictitating membrane is similar in duration to that of noradrenaline and dissimilar from the prolonged effect of amphetamine or ephedrine.

The different substances in this group while similar in the above respects were dissimilar in activity. Corbasil had about one-fifth, epinine about one-tenth, dihydroxyephedrine about one-twenty-fifth, and hydroxytyramine about one-fiftieth the pressor activity of noradrenaline in the spinal cat. The injection of 80 $\mu$g. corbasil, however, caused a mean contraction of the denervated membrane of only 34 mm. in 6 cats as compared

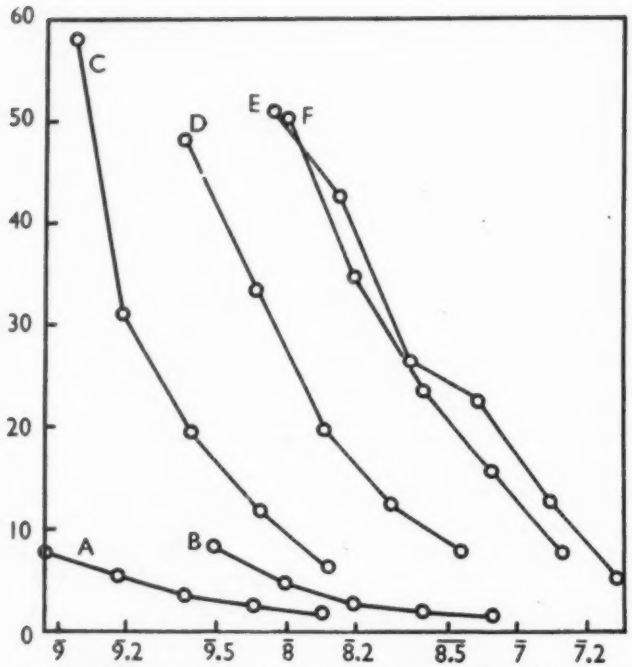



FIG. 7.—Ordinate is ratio of contractions in denervated and normal membranes. Abscissa is logarithm of dose in moles. *A:* adrenaline; mean of 25 cats. *B:* neosynephrine (*m*-Sympatol); 4 cats. *C:* noradrenaline; 25 cats. *D:* corbasil; 6 cats. *E:* dihydroxyephedrine; 4 cats. *F:* epinine; 6 cats. Increasing doses of the different substances were injected, the range of doses chosen being such as to give similar pressor effects for the different substances.

with the injection of 16 $\mu$g. noradrenaline which caused a mean contraction of 52 mm. in 25 cats. This smaller effect of corbasil on the nictitating membrane probably indicates a smaller affinity for the receptors on the membrane.

Adrenaline and neosynephrine were found to exert more effect on the normal membrane than noradrenaline or corbasil. Their effect on the denervated membrane was almost the same as that of noradrenaline.

## DISCUSSION

The observations which have been described may be considered in the light of the evidence that the enzyme amine oxidase is present in the nictitating membrane (Robinson, 1952) and that this enzyme declines in amount after removal of the superior cervical ganglion (Burn and Robinson, 1952). The observations made from the third day onwards when further change is slight will be considered first. The substances of the first group, which have less than two -OH groups in the ring, and no -OH group in the side-chain, either fail to stimulate the nictitating membrane after denervation or stimulate it feebly. Those with least effect on the denervated membrane have no -OH group in the ring and a $-CH_3$ group on the $\alpha$-carbon atom. These are amphetamine and its N-methyl derivative. The presence of one -OH group in the ring increases the action on the denervated membrane to some extent, and the removal of the $-CH_3$ group from the $\alpha$-carbon atom to give tyramine increases it still more.

Since these substances have only a small action or very little on the denervated membrane, it is evident that their action on the normal membrane is likely to be connected with the presence of the transmitter at the nerve ending and its destruction by the enzyme system located there. The identification of this enzyme is supported by the findings with amphetamine, N-methylamphetamine, and its *p*-hydroxy derivative, since those substances which have a $-CH_3$ group on the $\alpha$-carbon atom are known to inhibit amine oxidase. This suggests that the large effect of these substances on the normal membrane is indirect and is due to the action of the transmitter itself which, when the enzyme is inhibited, is no longer broken down.

This explanation, however, does not apply to tyramine and to $\beta$-phenylethylamine, which do not carry a $-CH_3$ on the $\alpha$-carbon. They do not inhibit amine oxidase. Tyramine is actually a substrate which is more rapidly destroyed by this enzyme than noradrenaline. There is evidence (Bülbring, unpublished) that tyramine can potentiate the action of sympathetic stimulation and of adrenaline on the nictitating membrane. We can therefore suppose that tyramine competes with the transmitter for the enzyme and in this way increases the effect of the transmitter on the nictitating membrane. Thus, with tyramine and probably also with $\beta$-phenylethylamine, the mechanism of the contraction of the normal membrane is essentially similar to that already described for amphetamine, but the contraction is of short duration.

We have now to consider the change in the response to these substances at the end of 24 hours, when all stimulate the denervated membrane more than the normal membrane.

In line with the foregoing argument the action on the denervated membrane suggests that at this time some transmitter is still being liberated from the postganglionic fibres. Beyond this, however, any explanation is entirely speculative. Before the final death of the fibres there might be an increased liberation of transmitter or a fall in the amount of enzyme or both.

It remains to consider the increase in the sensitivity of the denervated membrane to low doses of *p*-hydroxy-N-methylamphetamine and especially to tyramine which persists for weeks in many cats.

The low dose of tyramine must stimulate the denervated membrane directly and its smaller effect on the normal membrane may be explained by its destruction by the greater amount of enzyme present there. However, the denervated membrane seems peculiar in that the extent to which it can be stimulated by tyramine is limited, and, as the dose is increased, no further effect is produced. With *p*-hydroxy-N-methylamphetamine there is also a permanent increase in the sensitivity of the denervated membrane to the lowest doses, though this is much less conspicuous than with tyramine. This cannot be explained by increased destruction of this substance in the normal membrane by the enzyme, since the enzyme does not destroy it. Presumably, however, since it inhibits the enzyme it combines with it, and therefore a greater proportion will be free to act on the denervated membrane where the amount of the enzyme is smaller.

Thus the consideration of the action of substances in the first group can be summarized by saying that the effects they produce in the normal and denervated membranes are all explicable in terms of the presence of an amount of amine oxidase in the normal membrane which declines in the denervated membrane and in terms of the weak direct stimulant action these substances possess.

The substances in the second group differed from those in the first in having an -OH group on the carbon atom next to the ring. All these substances had much more action on the denervated membrane than the substances in the first group. The action of the compounds in the second group resembled that in the first group in being greater on the denervated membrane during the first 24 hours after removal of the ganglion and in being less on the denervated membrane (except in 2 cats with ephedrine) thereafter. From the second day onwards their greater action on the normal membrane may be explained as the sum of the direct action on the membrane and the indirect action due to a diminished destruction of the transmitter when the substance either competes with the transmitter for the enzyme or inhibits the enzyme. The effect of the two compounds with a $-CH_3$ group on the $\alpha$-carbon atom was more prolonged than that of the two compounds without this substituent. This is again consistent with the view that the enzyme concerned is amine oxidase.

The observation that $\beta$-phenylethanolamine and *p*-hydroxyphenylethanolamine acted for a longer time than $\beta$-phenylethylamine and tyramine respectively also accords satisfactorily with the biochemical evidence, since Blaschko (unpublished) found that the insertion of the -OH group in the side chain of tyramine slows down the rate of oxidation by amine oxidase.

The substances of the third group were with one exception catechol amines, having two -OH groups in the 3:4 position in the ring. Except for adrenaline and for neosynephrine (*m*-Sympatol) the effect of these compounds on the normal nictitating membrane was very small. When they were examined on the membrane 24 hours after removal of the ganglion, there was a small increase of sensitiveness ; when examined on the second and third day the increase was greater ; from the fourth day onward it became maximal and persisted.

These observations could be explained in terms of a diminution of amine oxidase only so far as the compounds without a $-CH_3$ group on the $\alpha$-carbon atom were concerned. The substances corbasil and dihydroxyephedrine are not destroyed by amine oxidase, and yet they behave in the same way as noradrenaline and epinine. There are various possibilities. One is that the pathway of destruction of noradrenaline does not involve the enzyme amine oxidase and that some other mechanism is concerned which deals with all catechol amines. Another is that despite the similarity of the actions on the membranes of noradrenaline and of corbasil, they are destroyed in different ways, noradrenaline by amine oxidase and corbasil by another mechanism.

The curves in Fig. 7 which show parallelism for noradrenaline and corbasil but not for noradrenaline and adrenaline might be taken to mean that while the destruction of noradrenaline follows the same path as that of corbasil it follows a different path from the destruction of adrenaline. However, when Burn and Robinson (1952) published their observations on the effect of denervation on the amount of amine oxidase they found a close correlation between the D:N ratio (the ratio of the contraction of the denervated membrane to that of the normal membrane) for noradrenaline and that for adrenaline. We have now made similar observations in 25 more cats. In each the D:N ratio was calculated for 4 $\mu$g. and for 8 $\mu$g. doses of both noradrenaline and adrenaline. There was much variation in these ratios. Thus those for 8 $\mu$g. noradrenaline varied from 3.0 to 42.0. The D:N ratios for the two substances were, however, found to be significantly correlated (at the 5% level of significance) both for the 4 $\mu$g. dose and for the 8 $\mu$g. dose. Hence it appears that the factors which account for the increased sensitivity to noradrenaline are the same as those which account for the increased sensitivity to adrenaline, and from

this it can be argued that the pathway of destruction of noradrenaline is the same as that for adrenaline. We have not yet compared noradrenaline and corbasil in this way.

Recently Schayer (1951) has published results of studying the fate of adrenaline injected into rats when labelled with $C^{14}$ in the N-methyl group and also when labelled on the $\beta$-carbon atom. He has come to the conclusion that "a reaction involving cleavage of the molecule at some point between the $\alpha$-carbon and the methyl carbon is a major route of adrenaline metabolism." This supports the view that adrenaline is destroyed by amine oxidase, and therefore, from the preceding evidence of the correlation of the D:N ratios, it seems likely that noradrenaline is also destroyed in this way. Such a conclusion leaves the destruction of corbasil and of dihydroxyephedrine as an unsolved puzzle.

The phenomena observed with substances like amphetamine and tyramine of the first group, and with substances like ephedrine of the second group, appear to be satisfactorily explained in terms of amine oxidase. This implies that when noradrenaline is liberated from the nerve ending as transmitter it is destroyed by this enzyme. The conclusion does not follow, however, that when noradrenaline is injected it is destroyed in the same way. The possibility remains that all the dihydroxy compounds when injected are inactivated as a result of changes in the benzene ring.

When Barger and Dale (1910) introduced the conception of sympathomimetic amines, the part played by the chemical transmitter at the sympathetic nerve ending and by the enzyme located there to destroy it could not be considered. We can now say that it is probable that substances can produce sympathomimetic effects (*a*) directly by combining with receptors as the transmitter, noradrenaline, does; (*b*) indirectly by inhibiting or deviating the enzyme which destroys the transmitter; and (*c*) by a combination of both these direct and indirect actions. This distinction of three kinds of sympathomimetic action was clearly indicated in the discussion of the action of ephedrine by Gaddum and Kwiatkowski (1938). The results obtained by comparing the effects of various amines on the normal and denervated nictitating membranes, a method introduced by Bacq (1937), make it possible to say what structural features place a compound having two carbon atoms in the side chain in one or other of these groups.

## Summary

1. A comparison has been made of the action of a number of sympathomimetic amines on the normal and denervated nictitating membranes of the spinal cat. Denervation consisted of removal of the superior cervical ganglion of one side, and observations were made at different times after the operation.

2. The amines tested could be classified by their action into three groups.

(*a*) Substances with not more than one -OH group in the benzene ring, and without an -OH group on the $\beta$-carbon atom, acted on the denervated membrane more powerfully than on the normal membrane only in the 24 hours following the operation. Later they caused little or no contraction of the denervated membrane. Their action appeared to be explained mainly by an inhibition or deviation of amine oxidase.

(*b*) Substances with not more than one -OH group in the benzene ring, but having an -OH group on the $\beta$-carbon atom, also acted on the denervated membrane more powerfully than on the normal membrane only in the 24 hours following the operation. Later they caused a considerable contraction of the denervated membrane but one which was smaller than that of the normal membrane. Their action appeared to be explained by a direct action on the membrane together with an inhibition or deviation of amine oxidase.

(*c*) Substances which were catechol amines had little action on the normal membrane and a much greater action on the denervated membrane from 48 hours after denervation onwards. Noradrenaline and corbasil were alike in their behaviour. The slight effect of these two substances on the normal membrane cannot be explained by supposing them to be rapidly destroyed by amine oxidase, since this enzyme does not attack corbasil.

We wish to thank Dr. D. J. Finney for testing the correlation between the D:N ratios for noradrenaline and adrenaline. This work was done by one of us (A. F.) during the tenure of a British Council Fellowship.

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# BLOWPIPE DART POISON FROM BORNEO

BY

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Mr. Rodney Needham recently sent from Sarawak a block of poison used on darts by the nomadic Pĕnan of Borneo. The block weighed 9 grammes and was wrapped in stout dried leaves. Mr. Needham said that it was a two years' supply for blowpipe darts. He described the method of using it. "A little of it is scraped into a large leaf folded to make a slight hollow; on to this is poured a little hot water, and the mixture is smeared on the darts for a length of about three inches from the point, more thickly at the actual point than elsewhere because usually little of it enters the animal. An alternative method is to pour a little hot water on to the block of poison itself, and to rub the darts along the grooves in it." The dart is about eight inches long.

We now describe a pharmacological examination of this poison.

## RESULTS

*Experiments on Blood Pressure.*—The block of poison was found to powder easily, and the powder was soluble when ground up in water or in saline to form a solution with a slight brownish tinge. The effect of injecting this solution intravenously into cats anaesthetized with chloralose was determined, recording the blood pressure and in some experiments the respiration also by Gaddum's method (1941). Fig. 1 illustrates the effect in one experiment. A volume of solution containing 0.4 mg. powder caused a rise in blood pressure and after 2 min. cardiac irregularity. Later, twice this dose caused a rise and then a fall of blood pressure which continued abruptly to zero. During the action of the first injection there was a slight slowing of the respiration from 28 to 22 per min., and during that of the second to 18 per min.

In all experiments both in cats and rabbits in which the respiration was recorded, it was observed that the usually abrupt fall of the blood pressure to zero occurred without any more serious change in the respiration than that in Fig. 1, so that it was clear that death did not result from respiratory failure. When the heart was examined after death the left ventricle was found to be tightly contracted and hard.

*Slow Intravenous Injection.*—The time elapsing between the injection of the poison and the arrest of the heart, the abrupt fall in blood pressure, and the arrest of the heart in systole all suggested that the poison had an action not unlike that of a glycoside of digitalis or strophanthus. We therefore studied the effect of the slow intravenous infusion of the poison in a solution containing 1–2 mg. in 40 ml., since when administered in this way the cardiac glycosides produce characteristic changes. Observations were made in rabbits under urethane anaesthesia and in cats under chloralose anaesthesia. Some parallel observations were made in cats using a solution of ouabain (crystalline strophanthin, from *Strophanthus gratus*).

An infusion of ouabain causes at first a progressive fall in the heart rate. A fall was observed with the arrow poison, though it was often small; thus in one cat the heart rate fell from 180 to 146 per min.; in a second it fell from 232 to 196 per min., and in a third from 196 to 180 per min. Later the heart rate rose and the beat became irregular as it does when ouabain is infused. During the later part of the infusion artificial

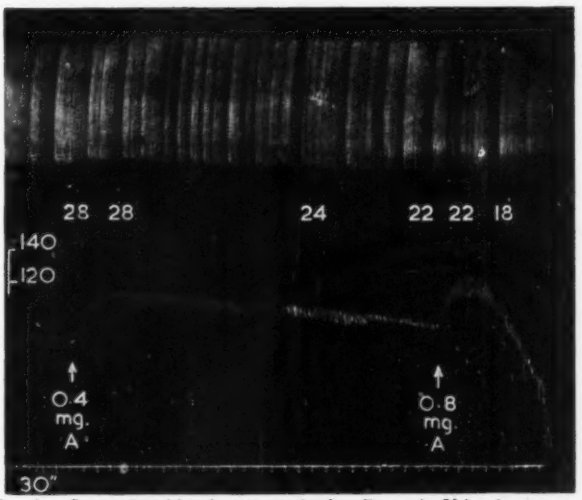


FIG. 1.—Cat under chloralose anaesthesia. Record of blood pressure (below) and respiratory movements (above) showing injection of 0.4 mg. and of 0.8 mg. of the poison (A). Note that 4 min. after the injection of 0.8 mg. the blood pressure fell to zero; the respiration became slower as indicated by the figures giving the rate per min. below the record.

respiration was usually given to ensure that death did not occur from respiratory failure. In some experiments the blood pressure fell more abruptly than in Fig. 1. In all experiments the arrest of the heart was the cause of death. The amounts of arrow poison calculated per kg. necessary to cause death in different animals are given in Table I, together with similar figures for ouabain.

TABLE I

LETHAL DOSE (MG. PER KG.) BY SLOW INTRAVENOUS INFUSION

| Arrow Poison | | Ouabain |
|---|---|---|
| Rabbit | Cat | Cat |
| 0·73 | 0·39 | 0·123 |
| 0·31 | 0·53 | 0·115 |
| 0·48 | 0·35 | 0·099 |
| 0·58 | 0·56 | 0·082 |
| | 0·66 | |
| | 0·46 | |
| Mean 0·52 | 0·49 | 0·105 |

The mean figure for ouabain, 0.105 mg. per kg., agrees with other published figures. Macdonald (1934) found a figure of 0.098 mg. per kg. for cats and Baker (1947) found a figure of 0.095 mg. per kg. for rabbits. Thus the toxicity of ouabain for rabbits is the same as that for cats ; the figures in Table I show that the toxicity of the arrow poison for rabbits was the same as that for cats. Moreover, the arrow poison from Borneo, although a crude preparation, contained activity equal to 20% of that of crystalline ouabain.

*Action on Isolated Cat Heart.*—Experiments were carried out on the isolated cat heart perfused with Locke's solution through the aorta by Langendorff's method. An example is given in Fig. 2. Perfusion with Locke's solution containing 5 mg. per litre of the poison caused the rate of beat to fall at first from 164 to 126 per min. After 7 min. the rate became very much faster, the amplitude of contraction then steadily diminishing. Finally the ventricles stopped and then contracted firmly, the auricles continuing to beat. The record is not unlike that obtained when a digitalis glycoside is similarly perfused.

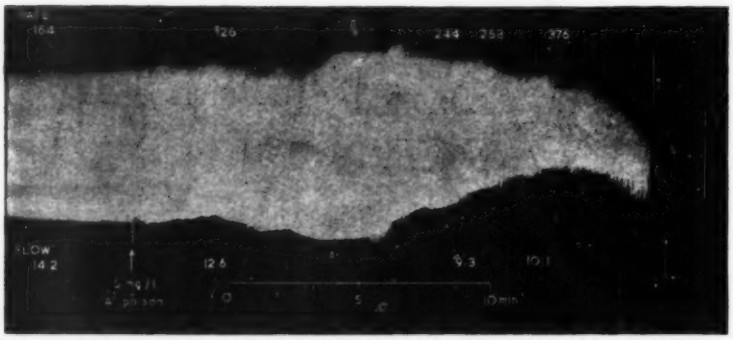


FIG. 2.—Isolated cat heart (Langendorff method). Effect of perfusing Locke containing 5 mg. of poison per litre. Figures above the record are the heart rate per min. Note the initial fall in rate and the later period of great quickening of rate. The beat was then arrested and the ventricle contracted firmly though the auricles continued to beat. Figures below the record are the coronary flow in ml. per min.

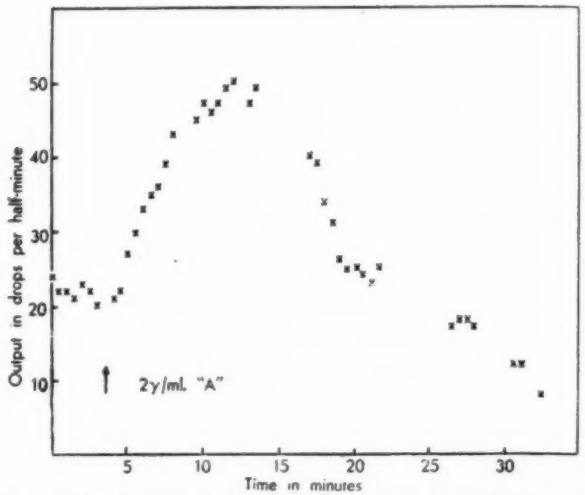


FIG. 3.—Frog heart perfused by Bülbring's method using Ringer containing one-quarter the normal amount of calcium. Ordinate: Output in drops per 30 sec. Abscissa: Time in min. Perfusion at the arrow with the poison (2 μg./ml.) caused a rise in the output resembling that caused by ouabain, when perfused in the same concentration.

*Action on the Isolated Frog Heart.*—In the observations so far recorded there was no evidence of any save a toxic action on the heart muscle. A substance which has the characteristics of a cardiac glycoside such as digoxin or ouabain should have an early beneficial action by which the force of the beat is improved and the output increased. This effect can be demonstrated very simply for ouabain by using the Bülbring (1930) preparation of the frog heart in which the inferior vena cava is cannulated for perfusion from a Marriotte bottle, and one aorta is cannulated in order to measure the output, the other being tied. When the heart is perfused with frog-Ringer containing one-quarter the normal amount of calcium, the change to a fluid containing in addition ouabain (2 μg./ml.) causes a large rise in output.

A series of seven experiments was therefore carried out with this preparation, using instead of ouabain the poison under examination in a concentration of 2 μg. poison per ml. Fig. 3 illustrates the effect observed, which closely resembles the effect obtained with ouabain (see Burn, 1952). Thus the poison resembles a cardiac glycoside such as ouabain in this important respect also.

*Toxicity for Mice.*—Although the glycosides of digitalis and strophanthus have a high toxicity per unit of body weight for cats and for rabbits, they have a much lower toxicity for mice. If the active principle in the poison was similar to one of the cardiac glycosides, then it should be found to have a low toxicity for mice. A comparison was therefore made between crystalline ouabain and the arrow poison on mice.

That the arrow poison had a low toxicity for mice was soon evident. Whereas the lethal dose for cats and rabbits was about 0.5 mg. per kg., that for mice was approximately 40 mg. per kg., an amount about 80 times as great. To compare the arrow poison with ouabain, several preliminary experiments were made, and then four experiments each on 40 mice were carried out. The results of the four experiments are given in Table II.

TABLE II
TOXICITY FOR MICE BY SUBCUTANEOUS INJECTION

| Exp. | Arrow Poison | | Ouabain | |
|---|---|---|---|---|
| | Dose (mg. per kg.) | Mortality | Dose (mg. per kg.) | Mortality |
| 1 | 40·0 | 12/20 | 14·0 | 13/20 |
| 2 | 40·0 | 12/20 | 12·5 | 10/20 |
| 3 | 27·5 | 11/20 | 9·5 | 11/20 |
| 4 | 19·0 | 2/20 | 6·5 | 7/19 |

The different experiments were done on different days and the mice were not equally sensitive on the different days. Thus in Exp. 3 the mice were more sensitive both to the arrow poison and to the ouabain, since a lower dose of both agents killed almost the same proportion of mice as in Exp. 2. However, a comparison could be made between the arrow poison and ouabain in each of the experiments; evidently the ouabain was about three times as toxic as the arrow poison, since ouabain in one-third the dose killed about the same proportion of mice. The experiments could also be combined, since of the mice injected with arrow poison (in Exps. 1, 2, and 3) 35 out of 60 were killed, and of the mice injected with ouabain 34 out of 60 were killed. These mortalities being the same, it could be said that the mean of the three doses of arrow poison, namely 35.8 mg., was equivalent to the mean of the three doses of ouabain, namely 12.0. Hence, the arrow poison had 33% of the toxicity of ouabain for mice; thus like ouabain it was much less toxic for mice than for cats and rabbits.

*Difference from Aconitine.*—When the first observations were made on the cat which showed that the arrow poison had a lethal action on the heart, it seemed possible that the active principle might resemble aconitine. The foregoing results appear to us to establish the close similarity of the arrow poison to ouabain, but it is perhaps worth while recording how it differs from aconitine. When this substance was injected by slow intravenous infusion into four cats under chloralose, the mean lethal dose was not much greater than that of ouabain; it was 0.145 mg. per kg. The effect on the respiration and the blood pressure was, however, very different from that of ouabain and from that of the arrow poison. When only 1–2 ml. of the solution had entered the cat the respiration was profoundly depressed and the blood pressure fell to a low level as seen in Fig. 4. Both blood pressure and respiration recovered later, and the cats died from the action on the heart, though the hearts were not always arrested in systole. Fig. 4 can be compared with Fig. 1, in which the arrow poison was injected. Experiments in which the arrow poison was slowly infused gave

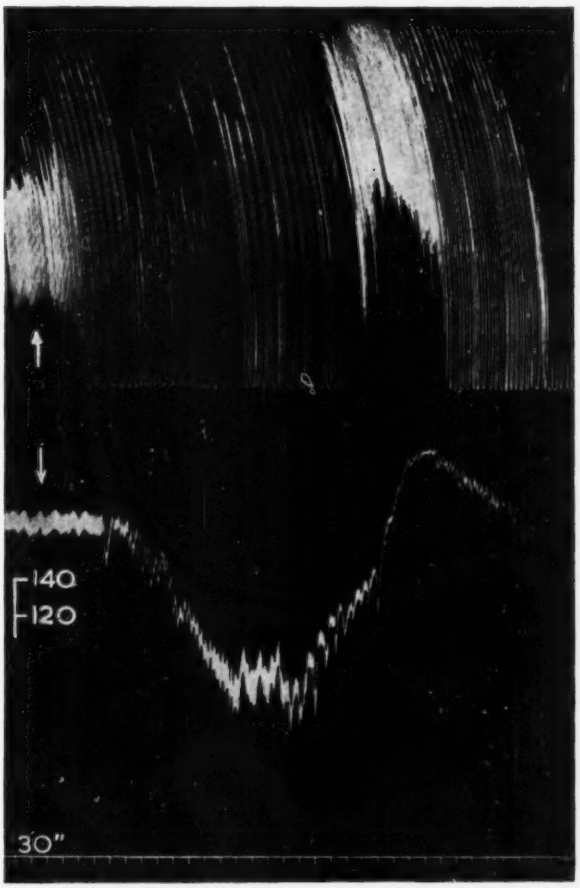


FIG. 4.—Cat: chloralose. Upper record is of respiratory movements, lower record is of blood pressure. At the arrow aconitine was given by slow intravenous infusion until the heart ceased to beat. Note the great slowing of the respiration and the fall of blood pressure, which did not occur when the arrow poison was similarly administered.

records which resembled that in Fig. 1, in that the respiration was only slightly slowed, the depth being unaffected, and that there was no fall of blood pressure except when the heart stopped.

The second difference between aconitine on the one hand and the arrow poison and ouabain on the other lay in the high toxicity of aconitine for mice. The dose of 0.5 mg. per kg. (s.c.) killed 10 out of 10 mice ; the dose of 0.4 mg. per kg. killed 12 out of 20 mice, while the dose of 0.3 mg. per kg. killed 2 out of 10 mice. Thus the LD50 was approximately 0.4 mg. per kg. for the mouse as compared with 0.145 mg. per kg. for the cat. When these figures are compared with the corresponding figures for the arrow poison, 40 mg. per kg. for the mouse and 0.49 mg. per kg. for the cat, they show that the arrow poison could not contain aconitine.

## Discussion

Mr. Rodney Needham has told us that he has seen the arrow poison prepared in North Borneo by making horizontal cuts into the bark of a tree, collecting the liquid which exudes, and evaporating it slowly in leaves above a fire. He thinks that the tree is the upas tree, *Antiaris toxicaria*, but he is uncertain of this. He has seen a monkey killed by one of the arrows. When the arrow first hit it, the monkey took little notice except that it pulled the arrow out of the wound and threw it away. After some minutes the monkey retched, though it did not vomit, and soon after it dropped from the tree dead.

Pelikan (1857) states that Brodie as long ago as 1811, and later Emmert, recognized that "upas-antiar" arrested the heart, and that Mudler in 1838 extracted the active principle antiarin. Crystals of this and also the extract, upas-antiar, were found by Pelikan to arrest the heart of the frog when the spinal cord had already been destroyed, from which he deduced that the action must have been exerted on the heart itself. Dybkonsky and Pelikan (1861) compared upas-antiar with digitaline on the frog and found that like the latter it had a direct toxic action on the heart. In 1901 antiarin was clearly recognized as a digitalis-like glycoside by Straub, who stated that it was the most powerful of these glycosides and was available in crystalline form ; he found that the isolated frog heart was arrested by 0.001 mg. Hedbom's paper in the same year gives a long list of earlier work. Trendelenburg (1909) found the range of critical concentrations for the arrest of the frog heart to be the same for antiarin as for k-strophanthin. Later work includes that by Ridley (1930) and by Chopra and De (1934). Both papers describe the active principles of *Antiaris toxicaria* as glycosides with a digitalis-like or strophanthus-like action, and refer to the sap of the tree as being used as an arrow poison in Malaya, Java, and Burma.

So far as we have been able to discover, none of the many papers refer to the poison used in North Borneo, and since the tree has not been identified we cannot say for certain that the poison we have examined came from *Antiaris toxicaria*. Our evidence, however, establishes the poison as containing a principle similar to ouabain, and presumably not much inferior to it in potency.

## Summary

The evidence described shows that the blowpipe dart poison used by the nomadic Pĕnan of Borneo is a preparation containing an active principle resembling a cardiac glycoside like ouabain, and that the preparation has 20%, measured in the cat or the rabbit, or 33%, measured in the mouse, of the toxicity of crystalline ouabain. The poison causes death by arresting the heart in systole, and has no obvious action on respiratory movements or the blood vessels. The poison further resembles ouabain in having a beneficial action on the output of the frog heart which is demonstrable when the heart is perfused with low-calcium Ringer's solution. The lethal dose of the poison for mice per kg. of body weight is about 80 times greater than the lethal dose for cats and rabbits, as is likewise the relation for ouabain. The fact that the toxicity of the poison is 20% of that of ouabain on the cat, but 33% of that of ouabain on the mouse, shows that the active principle is probably not ouabain itself. Such differences in relative toxicity for different species are characteristic of cardiac glycosides.

The work has been done under the direction of Professor J. H. Burn, to whom our thanks are due.

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# STIMULATION OF ISOLATED RABBIT AURICLES BY SUBSTANCES WHICH STIMULATE GANGLIA

BY

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Although it is generally accepted that the ganglia of the sympathetic fibres to the heart are situated at some distance from it, in the stellate ganglion, there have been experiments reported from time to time suggesting that there are ganglia in the heart itself capable of liberating appreciable quantities of an adrenaline-like substance.

An investigation into this question was conducted by Hoffmann, Hoffmann, Middleton, and Talesnik (1945). These workers showed that, in the presence of atropine, acetylcholine had an adrenaline-like action on mammalian hearts, producing under these conditions a stimulation of force and rate of contraction as well as an increase in the coronary flow. They found that this stimulant action of acetylcholine was abolished by nicotine, by curare, and by ergotamine. By testing the coronary perfusate on the frog heart, on the rectal caecum of the chicken, and on the rabbit's gut they demonstrated that an adrenaline-like substance was released into the coronary outflow when this stimulant action of acetylcholine was observed. They concluded that either sympathetic ganglia or chromaffin tissue is present in the heart and thought that these probably play a role in the regulation of its activity. Hoffmann and his coworkers did not speculate on the nature of the preganglionic fibres to these structures. McDowall (1946) found that the slowing and weakening of isolated hearts produced by acetylcholine in small doses were invariably followed by a period of increased activity. In a few hearts examined by him this inhibition was preceded by a few very forcible contractions. Like Hoffmann and his coworkers, McDowall found that, after atropine, acetylcholine produced stimulation of the isolated heart; but according to him the stimulation was usually confined to the force of contraction, though sometimes the rate was also increased. McDowall noted that this action of acetylcholine might simulate that produced by adrenaline on the heart in affecting the rate and force of contraction simultaneously. He found that after ergotoxine the stimulant action of acetylcholine on the atropinized heart was abolished or even reversed. McDowall, however, was unable to suppress the stimulation by adding paralysing doses of nicotine. He thought it unlikely that acetylcholine produced its stimulant action under these conditions by the stimulation of sympathetic ganglia in the heart.

During the course of examining some choline derivatives prepared by Dr. H. R. Ing in this laboratory, it was found that one of these had a nicotine-like action on the blood pressure of the cat; but when this compound was tested on the isolated rabbit auricle and the rabbit intestine it exerted a muscarine-like action. On studying the actions in the presence of hexamethonium, it was found that the apparent muscarine-like action disappeared and therefore was really a nicotine-like action. This discovery led to an investigation of the action of nicotine and other drugs on the isolated rabbit auricle.

## METHOD

Freshly dissected rabbit auricles were suspended in a bath of 40 ml. volume containing Ringer-Locke solution at 29° C., aerated with oxygen. Some preparations were kept overnight; they were placed in an evaporating dish containing about 10 ml. of Locke solution, covered with a wad of cotton-wool, and stored in the refrigerator at a temperature of 2–4° C. When testing the effect of nicotine it was found that several washes were necessary to remove all the nicotine afterwards. Care was taken to wash out the bath the same number of times after every application of a drug.

## RESULTS

When a dose of 0.4 mg. nicotine was applied to the isolated rabbit auricles a mixed effect was noted (Fig. 1). There was inhibition followed by some stimulation of the auricular beat. When the same dose of nicotine was applied in the presence of 10 $\mu$g. atropine, stimulation alone was seen.

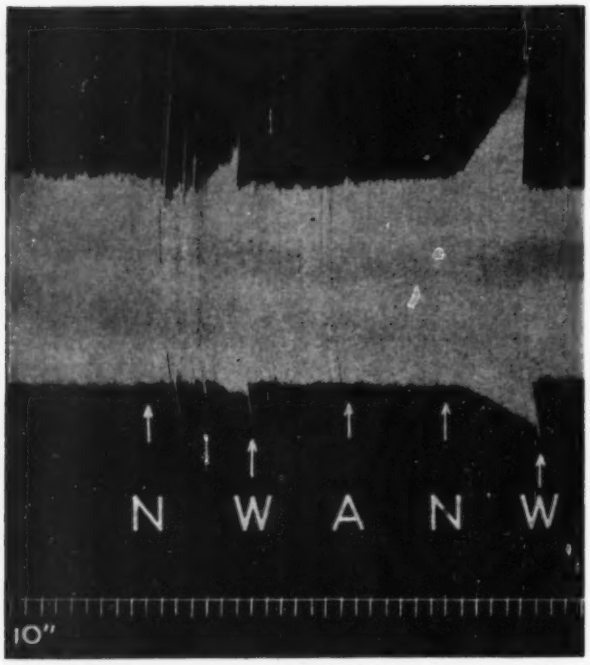


FIG. 1.—Spontaneous contractions of rabbit auricles in a bath of 40 ml. At N 0.4 mg. nicotine hydrogen tartrate was added. Note the mixed effect of inhibition and stimulation. After 1 min., at W, the bath was changed 8 times, during which the drum was stopped. At A 10 $\mu$g. atropine sulphate was added. In its presence the addition of nicotine as before caused stimulation only.

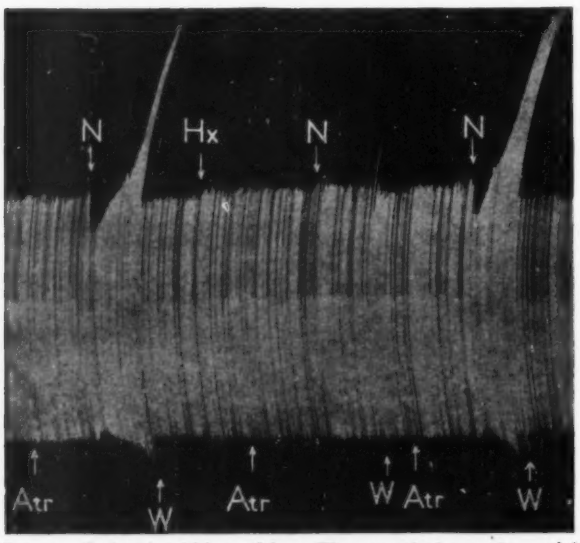


FIG. 2.—Isolated rabbit auricles. The record shows the mainly stimulant action of 0.8 mg. nicotine hydrogen tartrate (N) in the presence of 10 $\mu$g. atropine sulphate (Atr). At W the bath was changed 15 times. At Hx 5 mg. hexamethonium bromide was added, followed by 10 $\mu$g. atropine sulphate. Nicotine was then without effect. After washing out the bath, the stimulant action of nicotine returned.

In Fig. 2 is seen the effect of 0.8 mg. nicotine in the presence of 10 $\mu$g. atropine. There was initially a small inhibition followed by stimulation. On this preparation 0.4 mg. nicotine applied earlier had no effect. After washing out several times, 0.8 mg. nicotine was applied after atropine in the presence of 5 mg. hexamethonium. It is seen that hexamethonium suppressed the action of nicotine completely. On washing out the hexamethonium, nicotine again stimulated the auricles in the presence of atropine. When the same auricles were examined after they had been kept for about 18 hours at 2–4° C., a dose of 0.4 mg. nicotine, which had no effect on the previous day, stimulated the auricular beat. This action was again abolished by hexamethonium.

Fig. 3 shows the action of the phenyl ether of choline (kindly supplied by Dr. P. Hey) on the auricles. This substance has a strong nicotine-like action. In the presence of 50 $\mu$g. atropine, 0.2 mg. of the phenyl ether of choline caused an initial transient inhibition followed by a strong stimulation. As with nicotine this stimulant action was absent when hexamethonium was present in the bath. After washing out the hexamethonium the choline compound again caused stimulation.

Acetylcholine was similarly applied to the auricles (Fig. 4). In the presence of atropine a large dose of acetylcholine stimulated the auricles, and, as before, this action was not seen if hexamethonium was present. In a similar experiment Hoffmann and his co-workers also found that comparatively large doses of acetylcholine were necessary to produce stimulation after atropine. In Fig. 4 it is seen that as the hexamethonium was gradually washed out the stimulant action of acetylcholine returned.

It was conceivable that the stimulant action of nicotine and other drugs recorded above could be due to their acting more peripherally, like adrenaline, and that hexamethonium was able to block this peripheral action. To test this, a dose of 5 $\mu$g. adrenaline was applied to the auricles, and this dose was repeated in the presence of increasing concentrations of hexamethonium. A dose of hexamethonium as high as 80 mg. failed to suppress the action of 5 $\mu$g. adrenaline.

## DISCUSSION

The results which have been described show that nicotine, the phenyl ether of choline, and a large dose of acetylcholine exert in the presence of atropine a strong stimulant action on the isolated auricles of the rabbit, which is blocked by hexamethonium.

These findings recall the observations of Ambache (1951) on the gut. He found that botulinum toxin, which selectively paralyses the cholinergic nerve endings, reversed the action of nicotine

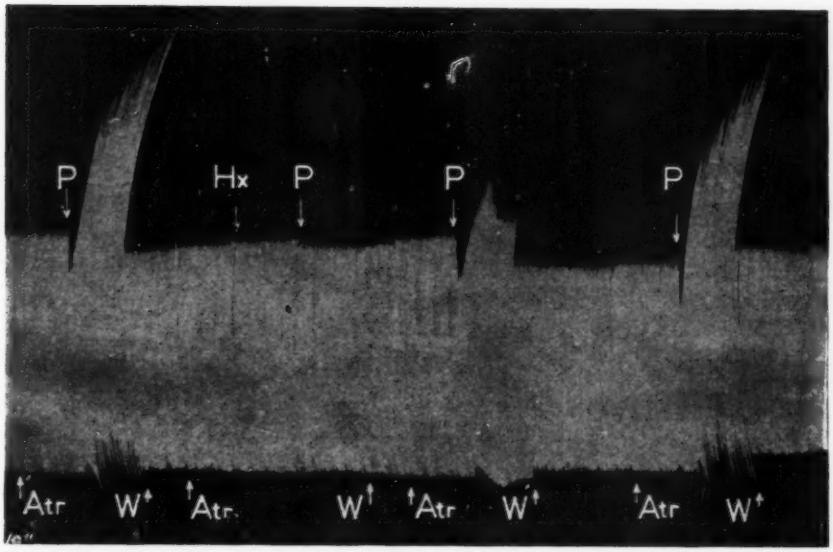


FIG. 3.—Isolated rabbit auricles. The stimulant action of 0.2 mg. phenyl ether of choline (P) in the presence of 50 $\mu$g. atropine sulphate. The action was absent when 2.5 mg. hexamethonium bromide (Hx) was added to the bath. The action returned gradually after washing out the hexamethonium.

on the gut, so that in its presence nicotine caused a relaxation. This effect was suppressed by hexamethonium and by paralysing doses of nicotine. Ambache and Edwards (1951) also showed that in the presence of atropine the action of nicotine on the gut was reversed. Hoffmann and his colleagues (1945) noted that it was not unusual for the atropinized rectal caecum of the chicken to relax after acetylcholine; they thought that this was probably due to nervous and chromaffin tissue in these preparations which released adrenaline when stimulated by acetylcholine or nicotine. Ambache postulated the presence of ganglion cells in the gut which, when stimulated, liberated an adrenaline-like substance. He thought that the pre-ganglionic fibres to these cells were vagal.

It is possible that the same situation exists in the heart. Dale, Laidlaw, and Symons (1910) showed that certain substances, nicotine, tropine, and curare, reversed the effect of vagal stimulation in the cat's heart. They thought that this was due to a selective paralysis rather than to a reversal of action. By eliminating all sympathetic nerves they came to the conclusion that the accelerator fibres were probably in the vagus. They considered that the presence of these accelerator fibres in the

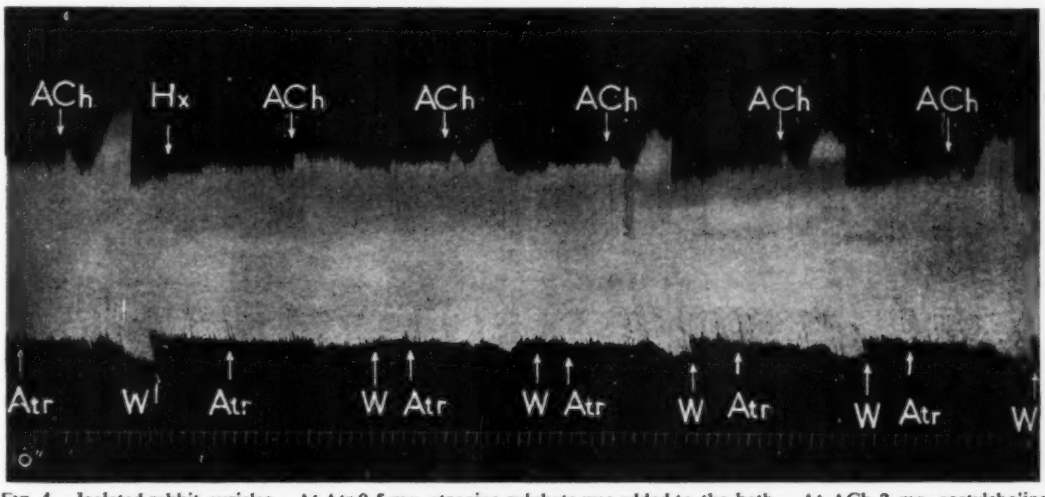


FIG. 4.—Isolated rabbit auricles. At Atr 0.5 mg. atropine sulphate was added to the bath. At ACh 2 mg. acetylcholine bromide was added. At Hx 5 mg. hexamethonium bromide was added. Note that the stimulant action of ACh in the presence of atropine was abolished by hexamethonium.

vagus might explain the readiness with which the cat heart escapes from inhibition by vagal stimulation.

One explanation, therefore, of the action of nicotine on the atropinized auricles is that there are ganglia or chromaffin tissue present which nicotine stimulates with consequent liberation of an adrenaline-like substance. This is in agreement with Ambache's hypothesis concerning the intestine. There are, however, other explanations, such as that nicotine may stimulate adrenergic nerves in continuity. Furthermore, actions of nicotine which are blocked by hexamethonium may not all be actions on ganglia.

## Summary

1. The application of nicotine to the isolated rabbit auricle causes a mixed inhibitory and stimulant action.

2. When nicotine, the phenyl ether of choline, or large doses of acetylcholine are applied to the isolated rabbit auricle in the presence of sufficient doses of atropine to block the " muscarine " effects, there is a marked stimulation of the auricular beat.

3. In the presence of hexamethonium the stimulant action of these drugs after atropine is suppressed, but the stimulation can be demonstrated again when the hexamethonium is washed out.

This work was done during a period of study leave from the University of Ceylon. It is with pleasure that I acknowledge my gratitude to Professor J. H. Burn for his guidance throughout the work.

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# THE SELECTIVE LOCALIZATION OF *p*-RADIOIODOPHENYLHYDROXYLAMINE IN RED CELLS: ITS RELATION TO METHAEMOGLOBINAEMIA

BY

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A recent communication reported the selective localization of radioactive material in the red cells and spleen of the rat after administration of *p*-iodophenylurethane and *p*-iodoaniline labelled with radioactive iodine (Crick and Jackson, 1952). From this work it was apparent that a common metabolic product was responsible for the localizing effect. Accordingly, *p*-iodophenylhydroxylamine ($IC_6H_4NHOH$) and *p*-iodonitrosobenzene ($IC_6H_4NO$) have been prepared in radioactive form and their distribution in rat tissues examined. The results presented in this paper show that these two substances possess a very high affinity for erythrocytes by virtue of their reaction with haemoglobin to form a stable complex, and either or both are likely metabolic products of iodophenylurethane and iodoaniline in the rat.

## METHODS

*Preparative.*—Bamberger (1895) appears to have been the first to prepare *p*-iodophenylhydroxylamine although no details were given. Lapworth and Haworth (1921) prepared the same compound by sulphide reduction of *p*-iodonitrobenzene and recorded the same melting point as the previous author (104–105° C.). We have found reduction of iodonitrobenzene with an aluminium-mercury couple in moist ether to be a superior and convenient method; in numerous preparations of iodophenylhydroxylamine by this method the melting point of the pure compound has been consistently 95–96° C. (decomp.).

### *Preparation of* p-*Iodonitrobenzene Labelled with Radio-iodine*

*p*-Nitroaniline (2 g.) was dissolved in hydrochloric acid (8 ml., sp. gr. 1.1) cooled in ice-water and diazotized with sodium nitrite (1.07 g.) in water (5 ml.). The solution was transferred to a beaker, and, with continued cooling, potassium iodide (2.4 g. in 5 ml. of water containing a suitable quantity of radioactive iodide) was added in the course of a few minutes. The dark brown mixture was allowed to stand for 1.5 hours at room temperature when the brown solid which had separated was filtered off and washed successively with water, potassium iodide (5%), sodium thiosulphate (5%), and water. After drying for 1 hour *in vacuo* at 100° C. the product was treated with charcoal in chloroform solution and crystallized by addition of alcohol. Yield, 2.0 g., m.p. 166–167° C.

*Preparation of Radioactive* p-*Iodophenylhydroxylamine.*—*p*-Iodonitrobenzene (1 g.) suspended in anaesthetic ether (50 ml.) was added to a freshly prepared aluminium-mercury couple (from 1 g. of aluminium foil) and the solution boiled for 40 minutes. The mixture was filtered into a round-bottomed flask (250 ml.), a little porous pot added, and the solvent rapidly removed *in vacuo* by direct connection to a water pump. The flask may be immersed in *warm* water to aid the process. The solid residue in the flask was left *in vacuo* for a further 15 minutes at room temperature and recrystallized from benzene or benzene-light petroleum (b.p. 40–60° C.), from which the iodophenylhydroxylamine separates in very light, colourless plates m.p. 95–96° C. (decomp.). Yield 0.4 g.

*Preparation of Radioactive* p-*Iodonitrosobenzene.*—*p*-Iodonitrosobenzene was originally prepared by Bamberger (1895) from *p*-iodophenylhydroxylamine by chromic oxidation and steam distillation; in our hands this method yielded poor results. The following procedure is simple and particularly suited to the preparation of the radioactive compound. *p*-Iodophenylhydroxylamine (0.25 g.) was dissolved in dry, anaesthetic ether (20 ml.), a mixture of dry silver oxide (prepared from hot silver nitrate solution with less than the equivalent requirement of barium hydroxide solution) and anhydrous magnesium sulphate (1 g. of each substance) added, and the mixture allowed to stand for 30 minutes with occasional shaking. An emerald green colour rapidly developed. After filtration the solvent was removed and the iodonitrosobenzene separated from a high-melting product (m.p. 195° C.) by crystallization from light petroleum. It forms green needles, m.p. 97–98° C. Yield, 0.15 g. The volatility of this compound necessitates especial care in handling the radioactive material.

In aqueous alcohol *p*-iodophenylhydroxylamine rapidly changes to 4:4′-di-iodoazoxybenzene m.p. 195° C., which is also produced in the preparation of the iodonitrosobenzene referred to above. Both iodophenylhydroxylamine and iodonitrosobenzene slowly undergo the same change at room temperature and should be kept at −20° C.

*Animal Techniques and Measurement of Radioactivity.*—For intravenous administration the iodophenylhydroxylamine was dissolved in a minimal quantity of ethanol, 2–3 volumes of water were added with shaking, and the solution used immediately. For intraperitoneal administration freshly prepared solutions in olive oil were used. The iodonitrosobenzene is insoluble in water and alcohol and appeared to decompose in olive oil. Its solubility in liquid paraffin was adequate for intraperitoneal use. The other techniques used have been previously described (Crick and Jackson, 1952).

## Results

*p-Iodophenylhydroxylamine* ($IC_6H_4NHOH$).—This substance, like phenylhydroxylamine, causes immediate and intense methaemoglobin formation when added to blood or washed red cells or injected intravenously into rats. The blood and tissue distributions of radioactivity produced by a single intravenous injection are shown in Fig. 1. There is a very rapid and specific localization of radioactivity (about 50% of the dose administered) in the erythrocytes and, with the exception of the spleen, no significant retention in other tissues. No appreciable fall in the level of activity in the blood occurs at this dose level during 24 hours after administration of the substance. A similar result is obtained after intraperitoneal injection (10 mg./kg. in olive oil), for in spite of the insolubility of the compound in water the effects appear very rapidly (Fig. 2); in a few minutes the animals pass into a state of collapse with severe methaemoglobinaemia. The duration of the latter is surprisingly short, visible methaemoglobinaemia having disappeared in about 2 hours, with complete recovery of the animals. In spite of this, the radioactive indicator shows that the drug is still present in some form within the red cells (Fig. 2).

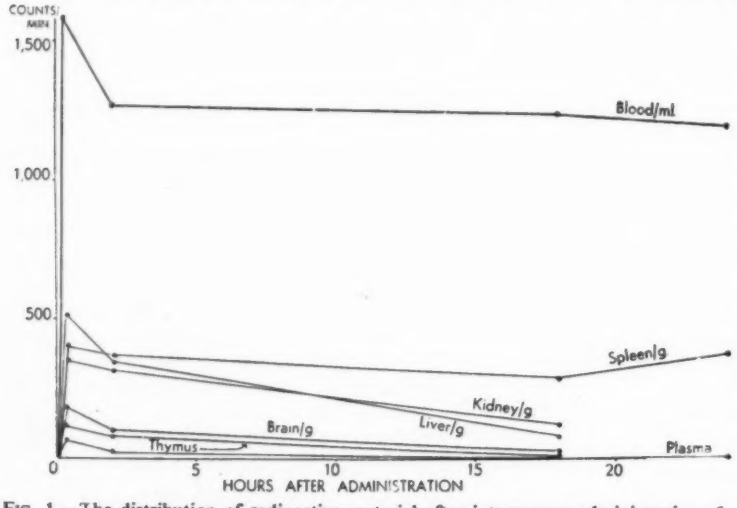



FIG. 1.—The distribution of radioactive material after intravenous administration of *p*-iodophenylhydroxylamine (5 mg./kg., specific activity 29 counts/μg./min.). Each point represents the mean value from a group of five rats.

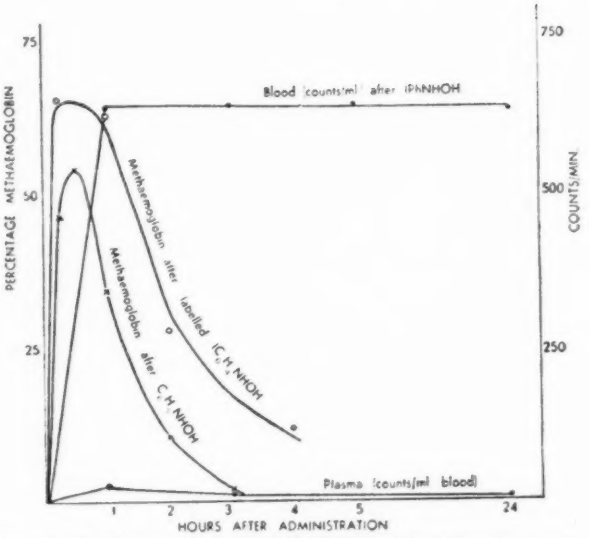



FIG. 2.—The intraperitoneal administration of iodophenylhydroxylamine and methaemoglobinaemia. Dose, 10 mg./kg., 10 counts/μg./min. Methaemoglobinaemia is rapidly induced by the drug but disappears quite quickly, whereas radioactive material is retained within the red cells. Iodonitrosobenzene produces essentially the same results as the iodohydroxylamine. Methaemoglobinaemia induced by an equivalent dose (5 mg./kg.) of phenylhydroxylamine is shown for comparison, and is less intense and persists for a shorter time.

The quantitative aspects of the reaction between iodophenylhydroxylamine and the cells has been studied in washed red cells in saline suspension by adding the iodo compound dissolved in a small quantity of aqueous alcohol and measuring the cell/saline distribution of radioactivity after varying periods of time (Table I). Maximal reaction occurs within a few minutes, the cells becoming intensely "methaemoglobinaemic," and the radioactivity in the cells cannot be dislodged by washing, nor does it emerge into the surrounding fluid on standing overnight at room temperature or 37° C. Over a wide range of concentration of iodophenylhydroxylamine the percentage radioactivity retained by the cells remained constant (Table I), but above 2,000 μg./ml blood haemolysis became significant

TABLE I

REACTION OF OXYGENATED ERYTHROCYTES WITH IODOPHENYLHYDROXYLAMINE AND IODONITROSOBENZENE

| Drug | μg. Added/ml. "Blood" (5 ml. Suspension) | Percentage Uptake After (hr.) | | | |
|---|---|---|---|---|---|
| | | 0.5 | 2 | 5 | 24 |
| IPhNHOH | 50 | 92 | 92 | — | — |
| | 100 | 92 | 90 | — | — |
| | 250 | 92 | 90 | 92 | 85 |
| | 375 | 90 | 91 | 93 | 91 |
| | 900 | 92 | 93 | 93 | 92 |
| | 2,000 | 97 | 90 | 92 | 36 |
| | 3,150 | 89 | 82 | 75 | —* |
| | 4,000 | 82 | 75 | 23 | —* |
| IPhNO .. | 700 | 94 | 93 | — | — |
| | 1,500 | 94 | 93 | — | — |

5 ml. citrated blood added to 20 ml. saline, centrifuged, the cells washed three times with saline and made up to the initial volume. 5 ml. of the suspension is thus equivalent to 1 ml. blood.

* Complete haemo'ysis.

and the corpuscular retention could not be assessed with accuracy. Estimation of the associated methaemoglobin formed in these experiments, using the method described by Evelyn and Malloy (1938), gave the results shown in Fig. 3. Obviously, small quantities of the iodo-compound are able to initiate the conversion of much haemoglobin to the ferric state, although the increase is

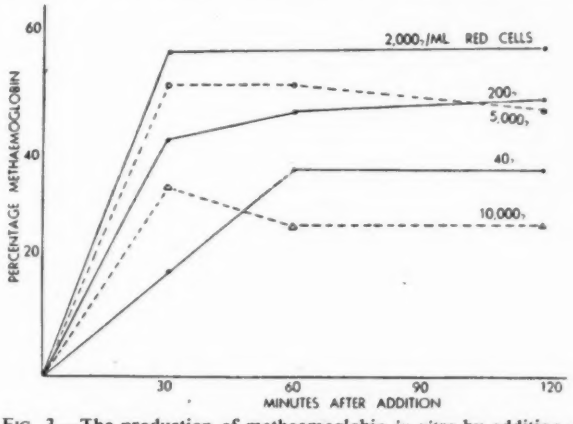



FIG. 3.—The production of methaemoglobin *in vitro* by addition of *p*-iodophenylhydroxylamine to washed rat erythrocytes in saline. A maximum of 60% methaemoglobin was produced by 2,000 μg./ml. red cells.

not maintained with increasing concentration of drug, a maximum of about 60% conversion being achieved. In spite of the rapid disappearance of the methaemoglobinaemia *in vivo* (Fig. 2), reversal was not achieved *in vitro* using radioactive cells washed repeatedly with saline and finally shaken with fresh plasma or saline, even with added glucose or cysteine, either at room temperature or at 37° C. This happened when the amount of radioactive compound corresponded to quantities of retained drug comparable to those achieved *in vivo* (e.g. 100 μg./ml. blood), where reduction of the methaemoglobin is complete in 2–3 hours. Reduction may easily be carried out with sodium hydrosulphite, however, and followed by shaking to oxygenate the pigment. Neither stage was associated with significant loss of radioactivity from the cells although haemolysis occurred during the reduction ; subsequent treatment with carbon monoxide also failed to dislodge the radioactive compound, the ratio of radioactivity to haemoglobin remaining constant (Table II).

TABLE II

Conversion of the methaemoglobin of labelled erythrocytes to haemoglobin and carboxyhaemoglobin does not remove radioactivity, although considerable haemo'ysis occurs. The ratio counts/haemog'obin remains steady.

| Suspension of Cells | Counts/ml. Suspension | mg. Haemoglobin/ml. Suspension | Counts/ Haemoglobin |
|---|---|---|---|
| Original .. .. | 18,950 | 77 | 250 |
| After hydrosu'phite | 6,560 | 29 | 230 |
| After carbon monoxide | 1,825 | 7 | 260 |

Experiments with cell suspensions in saline in which the haemoglobin was maintained in the reduced state by sodium hydrosulphite show that added iodophenylhydroxylamine can still be concentrated to some degree in the red cell (Table III), although this is now unaccompanied by any alteration in colour of the pigment. The concentration ratio (counts/ml. cells to counts/ml. saline) is less than one-tenth of that found for the oxygenated cells, so that the latter have a much greater affinity for the drug.

TABLE III

REACTION OF "REDUCED" ERYTHROCYTES WITH IODOPHENYLHYDROXYLAMINE AND IODONITROSOBENZENE

| Drug | μg. Added/ml. "Blood" (5 ml. Suspension) | Percentage Uptake After (hr.) | | |
|---|---|---|---|---|
| | | 0.5 | 1 | 2 |
| IPhNHOH.. | 100 | 30 | 31 | 21 |
| | 500 | 40 | 36 | 40 |
| | 1,000 | 41 | 43 | 41 |
| | 2,000 | 44 | 45 | 45 |
| IPhNO .. | 500 | 47 | 47 | 47 (overnight) |
| | 1,000 | 44 | 47 | 47 (overnight) |

Conditions of reaction as described in Table I.

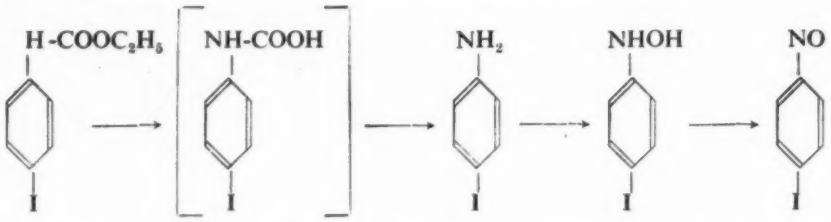
*p-Iodonitrosobenzene* ($IC_6H_4NO$).—Experiments have been carried out with this substance along similar lines to those described above, but were more restricted by the insolubility of the material. Administration of iodonitrosobenzene to rats by the intraperitoneal route in liquid paraffin solution (Fig. 2) was followed by the rapid onset of

collapse with intense methaemoglobinaemia. The distribution of radioactive material in the blood was very similar to that after iodophenylhydroxylamine. The *in vitro* reactions of iodonitrosobenzene with washed erythrocytes have been studied to a limited extent, and the concentration effects observed using both oxygenated and reduced cells were similar to those found after iodophenylhydroxylamine (Tables I and III).

It is indeed remarkable how rapidly these two insoluble compounds find their way into red cells from saline suspensions, even in relatively high concentrations.

## Discussion

The mechanism by means of which various aromatic nitro- and amino-compounds cause methaemoglobinaemia has been investigated for many years on account of its importance in relation to the use of such compounds in industry and therapeutics. Acetanilide has been especially singled out for study, and available evidence points to either phenylhydroxylamine or nitrosobenzene being directly responsible for the methaemoglobinaemia (Brodie and Axelrod, 1948 ; Greenberg and Lester, 1947 ; Bodansky, 1951). Only these substances, of likely metabolites, are really potent agents in inducing methaemoglobin formation directly in animals or when added to washed suspensions of red cells ; neither substance has been actually demonstrated to be a metabolite of acetanilide or aniline. This is not surprising in view of the instability of both phenylhydroxylamine and nitrosobenzene in aqueous media. The facility with which the *para*-iodo derivatives of phenylhydroxylamine and nitrosobenzene produce methaemoglobinaemia in spite of their relative insolubility in water, and the fact that they may carry a radioactive label, has enabled their metabolism and relation to methaemoglobin formation to be studied in more detail *in vivo* and *in vitro* with interesting results. We had already observed that the retention of radioactivity by red cells after exposure to *p*-iodophenylurethane or *p*-iodoaniline was associated with methaemoglobin formation *in vivo* only ; even so the abnormal pigmentation was a transient phenomenon whereas the radioactivity persisted within the cell (Crick and Jackson, 1952). Both *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene are much more potent agents than the iodourethane or iodoaniline and are also rapidly effective *in vitro*. The likelihood is that one or both of them are formed in the rat during the metabolism of the iodourethane and iodoaniline and is responsible for the localization of radioactivity within the erythrocyte. Thus:




The red cell component involved in the reaction is haemoglobin or methaemoglobin and the resultant union is very firm and appears to be independent of the state of the pigment, whether subsequently reduced, oxygenated, or converted to carboxyhaemoglobin. These findings are surprisingly different from the results previously reported by Keilin and Hartree (1943) using phenylhydroxylamine ($C_6H_5NHOH$), where the combination of this substance with haemoglobin (as followed spectroscopically) was stated to be very unstable and readily broken down by exposure to oxygen or carbon monoxide. They found that combination between haemoglobin and phenylhydroxylamine occurred in the proportion of 1 : 2 based on the iron content of the pigment, and that the new unstable compound possessed a characteristic absorption spectrum. The reactions of labelled iodophenylhydroxylamine and iodonitrosobenzene with crystalline haemoglobin and some of its derivatives are being examined in more detail, and an account will be published at a later date. It may be stated here that combination occurs in stoichiometric proportions to form compounds of considerable stability ; the globulin fragment of the haemoglobin is not involved in these reactions.

It is a curious observation that these two iodocompounds appear to be incapable of converting more than about 60% of the haemoglobin in erythrocytes to methaemoglobin *in vivo* or *in vitro* —an observation which has been previously made with phenylhydroxylamine (Lester, Greenberg, and Shukovsky, 1944). There are related compounds, on the other hand, like *p*-aminopropiophenone which are stated to cause practically complete conversion of the pigment *in vivo* in the dog to the ferric state (Vandenbelt, Pfeiffer, Kaiser, and Sibert, 1944 ; Bodansky and Gutman, 1947). The change to methaemoglobin appears to be a catalytic process in the lower concentration ranges of iodophenylhydroxylamine (Fig. 3) ; apparently the oxidation is complicated at higher concentrations

by the combination of either iodophenylhydroxylamine or iodonitrosobenzene with haemoglobin or methaemoglobin which may interfere with the spectroscopic estimation of the latter when the method of Evelyn and Malloy (1938) is used. The intermediate formation of a violet pigment may also be observed at higher concentrations before the final development of brown "methaemoglobin." This presumably is analogous to the unstable violet pigment referred to in the reaction between haemoglobin and phenylhydroxylamine (Keilin and Hartree, 1943).

The ability of phenylurethane, *p*-iodophenylurethane, and *p*-iodoaniline to induce methaemoglobinaemia in the rat depends upon the metabolic conversion of each of them into the hydroxylamine. Reference to Fig. 4 illustrates the results obtained after administration of these substances by the intraperitoneal route. Phenylurethane, although rapidly absorbed, does not apparently produce sufficient metabolite (aniline or phenylhydroxylamine) to induce methaemoglobinaemia, even at this high dose level. Both iodophenylurethane and iodoaniline, however, cause marked methaemoglobinaemia, from which one may infer that iodophenylhydroxylamine is more potent than phenylhydroxylamine in this respect, possibly by virtue of its greater stability, although both substances are unstable in solution. Moreover, the end-products are different; phenylhydroxylamine is converted into *p*-aminophenol and its *para*-iodo derivative into 4:4′-di-iodoazoxybenzene.

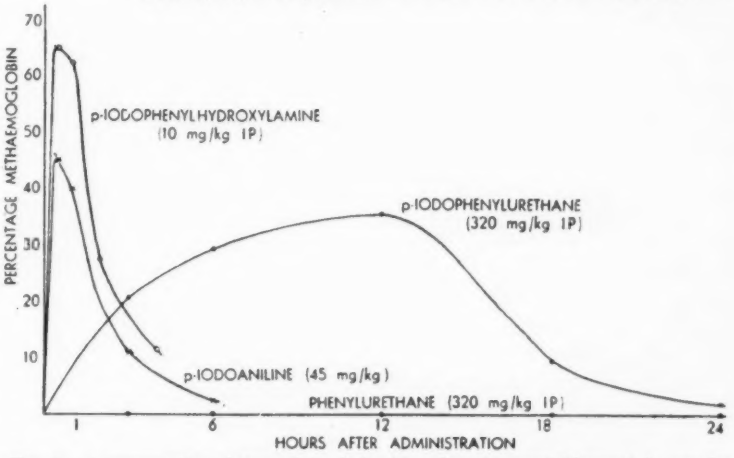



FIG. 4.—A comparative picture of the induction of methaemoglobinaemia in rats by *p*-iodophenylhydroxylamine (10 mg./kg.), *p*-iodoaniline (45 mg./kg.), *p*-iodophenylurethane (320 mg./kg.), and phenylurethane (320 mg./kg.), all by the intraperitoneal route. The sustained methaemoglobinaemia after the iodourethane is due to its more gradual metabolism involving the production of iodoaniline and then iodophenylhydroxylamine which is the active agent. Phenylurethane differs from its *para*-iodo derivative in not causing methaemog'obinaemia even at this high dose level. Presumably insufficient aniline and hence phenylhydroxylamine accumulates to cause any effect.

It is recognized that the methaemoglobin induced by chemical agents is easily reduced to haemoglobin by a reductase system present in the erythrocytes (for a review of the evidence see Bodansky, 1951). We have been unable so far to produce reversal in cells containing methaemoglobin induced by either iodophenylhydroxylamine or iodonitrosobenzene *in vitro* even when the amounts of these substances used produce corpuscular concentrations similar to those attainable *in vivo* where reversal readily occurs after a short time (Fig. 2). Nor was reduction promoted by addition of cysteine or glucose as has been the experience of other workers (Kiese, 1943, 1947; Bodansky, 1951). However, labelled methaemoglobinaemic cells are quickly reduced by sodium hydrosulphite without loss of radioactivity, although a fair amount of haemolysis occurs. Subsequent oxygenation of the cells or treatment with carbon monoxide produces the expected change in the cell pigment, also without loss of the radioactive label, which emphasizes the stability of the complex formed. Although phenylhydroxylamine produces the same sequence of events in the red cells as its *para*-iodo derivative, there appears to be no firm union between the former substance and haemoglobin. Thus preliminary treatment of washed red cells with as much as 6,000 $\mu$g./ml. of cells of phenylhydroxylamine failed to block subsequent reaction with iodophenylhydroxylamine. The intensely methaemoglobinaemic cells behave like normal cells in their ability to absorb and retain the radioactive material. The presence of the iodine atom in the aromatic ring is presumably responsible for the stability of the pigment complex.

## SUMMARY

1. The preparation of *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene labelled with radioactive iodine is described.

2. These substances localize specifically in the erythrocytes of rats and are firmly bound to the haemoglobin.

3. The associated methaemoglobinaemia which develops *in vivo* soon disappears leaving the radioactive material still bound within the cell. Reduction of methaemoglobinaemic cells produced *in vitro* was only achieved with the aid of sodium hydrosulphite, again without loss of radioactivity.

4. The radioactivity retained in cells is not removed by conversion of the haemoglobin to oxyhaemoglobin or carboxyhaemoglobin. The stability of the complex formed appears to be due to the *p*-iodo-substituent, since phenylhydroxylamine forms an unstable complex with haemoglobin.

5. The localization of radioactive material in erythrocytes observed after administration of *p*-iodophenylurethane or *p*-iodoaniline to rats is presumably due to the metabolic conversion of these two substances to *p*-iodophenylhydroxylamine and/or *p*-iodonitrosobenzene.

It is a pleasure to acknowledge the technical assistance of Mr. Robert Thompson and Miss Marion Bock in this work and the previous communication on *p*-iodophenylurethane.

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*Brit. J. Pharmacol.* (1953), **8**, 93.

# PROGUANIL—THE ISOLATION OF A METABOLITE WITH HIGH ANTIMALARIAL ACTIVITY

BY

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The existence of a metabolite, wholly or partly responsible for the antimalarial activity of "Paludrine" [proguanil, $N^1$-*p*-chlorophenyl-$N^5$-*iso*propyldiguanide (I ; R = H)], has been suspected for some years. Hawking and Perry (1948) showed that whilst the serum from animals dosed with proguanil was active *in vitro* against *Plasmodium gallinaceum*, the drug itself had no demonstrable effect on the exo-erythrocytic forms of the parasite grown in tissue cultures or on the blood forms of *P. cynomolgi* (see also Hawking, 1947, and Tonkin, 1946). Incubation of proguanil with minced liver was said to produce *in vitro* activity. Hawking and Perry therefore suggested that proguanil was modified in the body to form a product that was directly responsible for antimalarial activity. Madinaveitia and Raventos (1949) demonstrated that antimalarial drugs in general were adenosine antagonists and that their antimalarial activity was paralleled by the degree of this antagonism. The inhibition of the auriculo-ventricular block produced in guinea-pigs by the injection of adenosine was used to measure this effect, which was also manifest in heart-lung preparations and could therefore be examined under *in vitro* conditions. For instance, mepacrine was shown to antagonize adenosine both when administered to intact guinea-pigs and when added to normal guinea-pig blood in a heart-lung preparation. Proguanil, however, showed the effect in the intact guinea-pig only after some delay following its administration, and had no action on the heart-block in the isolated preparation. Therefore Madinaveitia and Raventos also postulated the existence in the body of an active metabolite of proguanil. This metabolic change might also be responsible for the greater antimalarial activity of proguanil when given orally than when given intravenously (Davey, 1946).

After a prolonged search for this hypothetical metabolite a highly active compound has eventually been obtained from the urine of rabbits dosed with proguanil. This communication describes in greater detail the isolation and characterization of this metabolite as recorded in a preliminary note (Carrington, Crowther, Davey, Levi, and Rose, 1951).

### *Isolation of an Active Proguanil Metabolite*

Most of the experiments have been carried out with rabbits. One of the first necessities was a method of estimating the drug in its original and metabolized forms. Spinks and Tottey (1946) described a method for the estimation of proguanil in tissues and body fluids which depended on the acid hydrolysis of the drug. In this method the samples were made strongly alkaline and the bases liberated were extracted by benzene and hydrolysed by an acid treatment. The *p*-chloroaniline so formed was diazotized and coupled with β-sulphato-ethyl-*m*-toluidine and the resultant azo compound estimated colorimetrically. The results obtained were in substantial agreement with the amount of proguanil that could be isolated from the benzene extract. In order to measure the total amount of material derived from *p*-chloroaniline, including unchanged proguanil, this method was modified by omitting the extraction with benzene and hydrolysing the whole urine, or other extract. For this purpose it was found more reliable to use alkaline hydrolysis under pressure.

Partly in order to confirm the validity of this analytical method, experiments were performed in which the bromo analogue of proguanil, $N^1$-*p*-bromophenyl-$N^5$-*iso*propyldiguanide, labelled with radio-active bromine, was administered to rabbits. The information obtained from counts on various urine fractions was of assistance in the detection and isolation of metabolites. Details of these experiments will be reported in a later communication.

Some of the samples taken at various stages in the extractions were too toxic for biological assay but whenever possible antimalarial activity against

*P. gallinaceum* in chicks was determined and a rough measure of the relative concentration of active material was deduced by comparison with the *p*-chloroaniline content of the samples.

Most effort was directed towards the examination of the urine from the treated animals. After the addition of lead acetate to the urine, in order to precipitate much unwanted material, the greater part of the therapeutically active fraction remained in solution and was then almost completely extractable into butanol. After distilling off the butanol, dissolving the residue in water and making strongly alkaline, part of the material yielding *p*-chloroaniline on hydrolysis could be extracted by ether, but part remained in the aqueous phase. The activity of the ether-soluble bases was shown to be due solely to unchanged proguanil. Estimations of the antimalarial activity remaining in the aqueous phase were at first erratic until it was realized that the active substance was unstable under alkaline conditions. Neutralization was effected, thereafter, as early as possible, the active substance extracted with butanol, and the solvent again replaced by water. Addition of picric acid was found to precipitate all the active principle as its picrate. The yield of picrate calculated to an assumed molecular weight of 500 was 1–3% based on the proguanil administered to the rabbits.

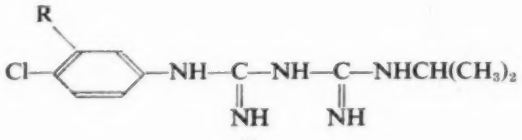
The faeces of the rabbits were also examined. After an ether treatment which did not remove any derivatives of *p*-chloroaniline, the faeces were extracted with methanol. The methanol solution was evaporated and *p*-chloroaniline derivatives were dissolved in dilute acid. This solution could then be treated as for the urine. In this way, a further 1% of picrate identical with that from urine was obtained.

The yield from the urine of human volunteers dosed with proguanil was about 5%.

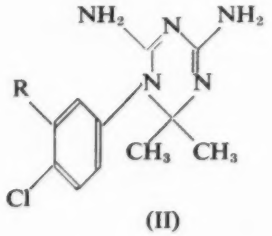
The picrate was readily converted into the free base, which was shown by our colleague Dr. D. G. Davey to be about ten times as active as proguanil against a blood-induced infection of *P. gallinaceum* in chicks. Like proguanil it was also effective against exo-erythrocytic forms of the parasite. Further, its activity was much reduced against proguanil-resistant strains of the parasite.

The metabolite has been shown to be 4:6-diamino-1-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine* (II ; R=H), and it has been found possible to synthesize this compound by a method which has proved general for dihydrotriazines of this type. Details of the synthesis together with an account of the work which established the structure of the metabolite will be published elsewhere.

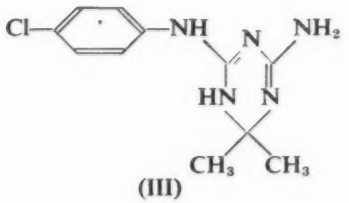
On treatment with alkali the metabolite was isomerized to give 4-amino-6-*p*-chloroanilino-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine (III), which was first prepared by Birtwell, Curd, Hendry, and Rose (1948), and which was inactive against *P. gallinaceum*. In acid solution the metabolite underwent facile hydrolysis to *p*-chlorophenyldiguanide (IV). The isolation of (III) and (IV) from the urine of monkeys dosed with proguanil has been reported by Crounse (1951). Under carefully controlled conditions we have only been able to isolate small amounts of (III) from rabbit urine, and it appears likely that these two "metabolites" arose as secondary products during Crounse's experiments.



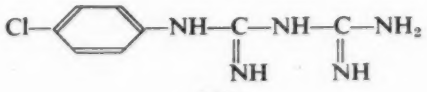
(I)



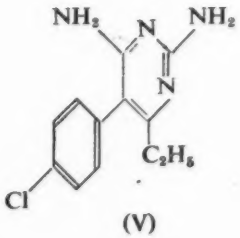
(II)



(III)



(IV)



(V)

* Patent protection pending.

Since as already indicated the antimalarial activity of the dihydrotriazine (II ; R = H) appeared to be of a very similar type to that of proguanil itself, it was now possible to conclude that proguanil owed its potency to the formation of the metabolite ; it was therefore to be expected that other active diguanides would undergo a similar metabolic change. To confirm this view the most potent compound of the diguanide series, $N^1$-3 : 4-dichlorophenyl-$N^5$-*iso*propyldiguanide (I ; R = Cl) (Curd, Davey, Hendry, and Rose, 1950 ; Crowther, Curd, Davey, Hendry, Hepworth, and Rose, 1951), was selected for study and administered to rabbits. The urine was extracted in the same way as before. The picrate finally isolated was identical with the picrate of a synthetic specimen of 4 : 6-diamino-1-(3 : 4-dichlorophenyl)-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine* (II ; R = Cl). To illustrate the relationship in activity between the dihydrotriazines and the corresponding diguanides it might be mentioned here that this member of the series (II ; R = Cl) was approximately 10 times as active against *P. gallinaceum* as the proguanil metabolite (II ; R = H) and therefore about 100 times as active as proguanil itself.

## Experimental

Rabbits were dosed orally with a 1% solution of proguanil hydrochloride at the rate of 50 mg./kg. once daily. No toxic symptoms were manifest at this dose, but at 70 mg./kg. delayed deaths occurred. No significant rise in glucuronide or ethereal sulphate in the urine was detected. In fresh urine the amount of diazotizable amine was negligible, but measurable amounts appeared on prolonged storage. This was true of many of the extracts. Proguanil itself was stable under these conditions.

*Determination of* p-*Chloroaniline Found on Hydrolysis of Urine Fractions.*—The samples were diluted with 0.5N-sodium hydroxide to contain, after hydrolysis, 20–200 μg./ml. of *p*-chloroaniline. Extracts in water-immiscible solvents were first evaporated to dryness. The diluted solution (3 ml.) was heated in a sealed ampoule in a pressure vessel at 120–125° C. for 16–20 hours. (Longer heating of urine samples under these conditions gave no increase in the estimated amount of *p*-chloroaniline.) After cooling, two 1 ml. samples were withdrawn and each diluted with 2 ml. of 0.5N-hydrochloric acid. Both were diazotized and one was coupled with β-sulphato-ethyl-*m*-toluidine (Spinks and Tottey, 1946). The other served as a blank control. The amount of *p*-chloroaniline was estimated colorimetrically, comparison being made with the curve constructed from results obtained with standard solutions of *p*-chloroaniline. The accuracy of the method under routine conditions was not high, but it proved adequate for following fractionation procedures and it was possible to obtain a reasonable material balance after a series of urine treatments.

Acid hydrolysis to *p*-chloroaniline was substantially complete in 4 to 6 hours, but erratic results were obtained in extracts containing high concentrations of salts and especially in the presence of lead.

*Isolation of 4 : 6-Diamino-1-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine (II ; R = H) from the Urine of Rabbits Dosed with Proguanil.*—Powdered lead acetate was added to the urine (e.g., 10 l.) from several rabbits until further addition caused no more precipitation. (During collection urine was stored at 0–5° C. over chloroform.) The suspension was filtered through Kieselguhr. The filtrate was extracted with butanol (3 × 3 l.). The combined extracts were evaporated *in vacuo* to a volume of 0.5–1 l. Water (about 2 l.) was added and the solution again evaporated *in vacuo*, to remove the butanol, to a volume of about 1 l. The solution was made faintly acid with hydrochloric acid and filtered from the precipitated lead chloride. Hydrogen sulphide was passed into the filtrate and the lead sulphide was removed. (When the acid treatment was omitted and all the lead was precipitated as the sulphide much loss of material occurred through irreversible adsorption.) The solution was freed from excess hydrogen sulphide by a current of air, and basified with concentrated sodium hydroxide solution, the temperature being kept at 0–5° C. by the addition of ice. The solution was extracted with ice-cold ether (300 ml.). The combined ether solutions (A) were reserved for later examination. The aqueous part was cooled by the addition of ice, neutralized with hydrochloric acid, and shaken with butanol (3 × 400 ml.). The butanol extracts were combined and washed with a minimum of dilute sodium hydroxide solution to remove any phenols present. Dilute hydrochloric acid was added gradually to the butanol part with shaking until both phases were neutral. The butanol layer was separated, evaporated *in vacuo*, water was added, and the solution again evaporated. The aqueous solution (100–200 ml.) thus obtained, which had high antimalarial activity, was treated with saturated alcoholic picric acid (10–20 ml.) and stirred. After standing overnight the picrate which had separated was collected and crystallized from ethanol to give canary-yellow needles of 4 : 6-diamino-1-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine picrate, m.p. 206° C. (Found : C, 42.3 ; H, 3.65 ; N, 22.8, 22.75 ; Cl, 7.3. $C_{11}H_{14}N_5Cl$, $C_6H_3O_7N_3$ requires C, 42.5 ; H, 3.5 ; N, 23.3 ; Cl, 7.4%).

The mother-liquor from the picrate still contained about 10% of the material hydrolysable to *p*-chloroaniline present before the separation of the picrate. It appeared to have little or no antimalarial activity and was not further investigated.

The crude picrate (367 mg.) suspended in 2N-hydrochloric acid (12 ml.) was exhaustively extracted with ether. The aqueous part was filtered through decolorizing carbon and cooled in ice. 10N-Sodium hydroxide was added drop by drop until no more solid

* Patent protection pending.

separated. The crystals were collected and washed with a very little ice-cold water, and dried *in vacuo* over phosphorus pentoxide; 114 mg., m.p. 144° C. The base was dissolved in chloroform (10 ml.), previously saturated with water, and filtered. Ether (5 ml.) and light petroleum (b.p. 40–60° C.) (5.5 ml.) were added to precipitate colourless prisms of the base (II; R=H); 56.4 mg., m.p. 143° C. (A repeat experiment gave m.p. 146° C.). (Found: C, 52.1; H, 5.55; N, 26.8, 27.0. $C_{11}H_{14}N_5Cl$ requires C, 52.5; H, 5.6; N, 27.8%). Ultraviolet absorption in 0.01N-hydrochloric acid: $\lambda$ max., 240 m$\mu$; log. $\varepsilon$, 4.09.

The base was soluble in cold water, being reprecipitated on the addition of sodium hydroxide, soluble in ethanol, and butanol. It was insoluble in ether, benzene, and dry chloroform and gave no complex on treatment with ammoniacal copper sulphate solution.

It was fully active against a blood-induced infection by *P. gallinaceum* in chicks when given orally at 0.01–0.025 mg./50 g., b.i.d. × 3½.

The ether extract (A) from several batches of the urine concentrate was evaporated to a small bulk and extracted with dilute hydrochloric acid. The aqueous part was neutralized by aqueous ammonia. The solid precipitated was collected, washed with water, and dried. It consisted of $N^1$-*p*-chlorophenyl-$N^5$-*iso*propyldiguanide hydrochloride (proguanil hydrochloride) (I; R=H) (cf. Spinks and Tottey, 1946). The filtrate was made strongly alkaline by the further addition of ammonia. An aqueous solution of copper sulphate was added to precipitate the rest of the proguanil as its copper complex. The mixture was filtered. There were no other detectable bases in the precipitate when freed from copper. The total recovery of proguanil represented about 10% of the administered drug. Hydrogen sulphide was passed through the filtrate, the precipitated copper sulphide removed, and the solution, which was devoid of antimalarial activity, was treated with picric acid. The precipitated picrate was collected and crystallized from ethanol to give orange-yellow needles in small amount of 4-amino-6-*p*-chloroanilino-1:2-dihydro-2:2-dimethyl-1:3:5-triazine picrate, m.p. 235° C. alone and mixed with an authentic specimen. Crounse (1951) gave m.p. 238° C.

*Isolation of (II; R=H) from the Faeces of Rabbits Dosed with Proguanil.*—The faeces were collected, air-dried, ground to a powder with anhydrous sodium sulphate, and exhaustively extracted with ether. Negligible amounts of *p*-chloroaniline derivatives were removed. The solid was treated with methanol by percolation and the extract was evaporated *in vacuo* to leave an aqueous solution and some tarry material. The residue was triturated repeatedly with dilute hydrochloric acid and the extracts were treated as in the isolation of the metabolite from urine from the stage at which the lead salts had been removed. The crude picrate was crystallized from ethanol to give canary-yellow needles, m.p. 206° C. undepressed on admixture with the picrate of (II; R=H). Ultraviolet absorption after conversion to a solution of the hydrochloride, in 0.01N-HCl: $\lambda$ max., 240 m$\mu$; log. $\varepsilon$, 4.07. The antimalarial activity was comparable with that of the metabolite obtained from the urine. The yield of the picrate was about 1% based on the administered proguanil.

*Isolation of (II; R=H) from the Urine of Human Volunteers Dosed with Proguanil.*—Two volunteers took a total of 6 g. of proguanil hydrochloride at the rate of 1 g. per day each for 3 days. The pooled urine was treated exactly as described for rabbit urine above. The yield of crude picrate of (II; R=H) was 608 mg. representing 5% based on the administered dose. The picrate, crystallized from *iso*propanol, had m.p. 209–212° C. and did not depress the m.p. of the picrate of (II; R=H). It was converted into the base which was crystallized from moist chloroform, ether and light petroleum (b.p. 40–60° C.) to give pure (II; R=H), m.p. 145° C., undepressed in admixture with authentic material. The base showed the expected antimalarial activity.

*Isolation of 4:6-Diamino-1-(3:4-dichlorophenyl-1:2-dihydro-2:2-dimethyl-1:3:5-triazine (II; R=Cl) from the Urine of Rabbits Dosed with $N^1$-3:4-Dichlorophenyl-$N^5$-iso*propyldiguanide *Hydrochloride.*—The drug was given at a dose of 30 mg./kg. once daily. Higher doses caused a high proportion of deaths amongst the rabbits. The urine was extracted as in the proguanil experiments. The yield of crude picrate corresponded to 0.75% based on the administered diguanide. Crystallization from aqueous 2-ethoxyethanol gave canary-yellow needles of the picrate of (II; R=Cl), m.p. 197–198° C. alone and mixed with an authentic synthetic sample. Ultra-violet absorption after conversion to a solution of the hydrochloride, in 0.01N-HCl: $\lambda$ max., 240 m$\mu$; log. $\varepsilon$, 4.09, in 0.1N-NaOH (after heating at 100° C. for 20 minutes in 0.1N-NaOH): $\lambda$ max., 260 m$\mu$; log. $\varepsilon$, 4.25. [The corresponding figures for an authentic synthetic sample were, in 0.01N-HCl: $\lambda$ max., 240 m$\mu$; log. $\varepsilon$, 4.10, in 0.1N-NaOH (after heating at 100° C. for 20 minutes in 0.1N-NaOH): $\lambda$ max., 262 m$\mu$; log. $\varepsilon$, 4.23.]

## Discussion

Several workers have attempted to elucidate the mode of action of proguanil by examining the effect of *p*-aminobenzoic acid and of pteroylglutamic acid on the properties of the drug (for references see Greenberg and Richeson, 1951). With the isolation of a highly active metabolite, however, coupled with the earlier reports that proguanil had no antimalarial activity *in vitro*, it now seems clear that the inhibition of the bactericidal action of proguanil on *Lactobacillus casei in vitro* by pteroylglutamic acid (Falco, Hitchings, Russell, and VanderWerff, 1949) has no bearing on the antimalarial effect of the drug, nor indeed have the results of any work *in vitro*. On the other hand, the inhibition of the action of proguanil *in vivo* against *P. gallinaceum* by pteroylglutamic acid (Greenberg, 1949) and against *P. berghei* by

*p*-aminobenzoic acid (Thurston, 1950) must have some significance concerning the mode of action of the metabolite. It is unlikely that either pteroylglutamic acid or *p*-aminobenzoic acid interferes with the formation of the metabolite since the former had no effect on the action of proguanil against ***P. berghei*** (Thurston, 1950) and the latter had no effect on the action against ***P. gallinaceum*** (Bishop and McConnachie, 1948).

The similarity in structure between proguanil and the antimalarial 2:4-diamino-5-aryloxypyrimidines has been pointed out by Falco, Hitchings, Russell, and VanderWerff (1949). There is a much more remarkable resemblance between the structure of the proguanil metabolite (II; R=H) and the most active of the 2:4-diamino-5-aryl-pyrimidines (V) of Russell and Hitchings (1951) and Falco, Goodwin, Hitchings, Rollo, and Russell (1951).


## SUMMARY

1. A metabolite of "Paludrine" (proguanil) has been isolated from the urine and faeces of rabbits and from the urine of human volunteers dosed with the drug. The metabolite, 4:6-diamino-1-*p*-chlorophenyl-1 : 2-dihydro-2 : 2-dimethyl-1 : 3 : 5-triazine (II; R=H) had an activity about 10 times that of proguanil against blood-induced infections of *P. gallinaceum* in chicks and was also active against the exo-erythrocytic forms of the parasite.

2. Only insignificant amounts of its isomer, 4-amino-6-*p*-chloroanilino-1 : 2-dihydro-2 : 2-dimethyl-1:3:5-triazine (III), were present in the rabbit urine.

3. 4:6-Diamino-1-(3:4-dichlorophenyl)-1 : 2-dihydro-2:2-dimethyl-1:3:5-triazine (II; R=Cl), isolated from the urine of rabbits dosed with $N^1$-3:4-dichlorophenyl-$N^5$-*iso*propyldiguanide, was about 100 times as active as proguanil against the blood forms of *P. gallinaceum* in chicks.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# THE EFFECT OF ADRENALINE AND NORADRENALINE INFUSIONS ON RESPIRATION IN MAN

BY

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During the course of investigations on the cardiovascular responses to intravenous infusions of adrenaline and noradrenaline in man an increase in the rate and depth of the respiration was frequently observed, especially during the first few minutes of the infusion period. This was usually accompanied by a feeling of tightness in the chest.

The stimulating action of small doses of adrenaline on the respiration in unanaesthetized man (Tompkins, Sturgis, and Wearn, 1919; Lyman, Nicholls, and McCann, 1923; Cori and Buchwald, 1930; Courtice, Douglas, and Priestley, 1939) and animals (Boothby and Sandiford, 1923) and in anaesthetized animals (Nice, Rock, and Courtright, 1914; Nice and Neill, 1924; Wright, 1930) is well known. Reale, Kappert, Skoglund, and Sutton (1950) reported an increased rate and depth of respiration with intravenous noradrenaline in man, but a direct comparison of the effects of the two drugs on the respiration does not appear to have been made.

In the present investigation the influence of adrenaline and noradrenaline on the respiratory pattern and gaseous exchange was examined in the conscious human subject.

## METHODS

The subjects were examined two to three hours after a light breakfast. The laboratory was temperature-controlled at 20–23° C., and the subject lay on a couch with face-mask and stethograph in position for at least 30 minutes before any observations were made.

The experiments were divided into two groups. In the first, records were made of the respiratory pattern while the oxygen consumption was determined during intravenous infusions of adrenaline and noradrenaline. Records of the forearm blood flow were made at the same time to correlate the respiratory changes with the cardiovascular responses to the infused drugs. In the second group of experiments the changes in alveolar $CO_2$% were recorded in addition to the respiratory movements.

The rubber face-mask covered only the nose and was carefully fitted to avoid leaks. It was used in preference to a mouth-piece because it was more comfortable and interfered to a minimal extent with the resting pattern of respiration. Tracings of the rate and depth of respiration were obtained with a float-recorder connected to two stethographs, one around the chest and the other around the abdomen (Shepherd, 1951; Dornhorst and Leathart, 1952). Forearm blood flow was measured with a venous occlusion plethysmograph filled with water at 33–35° C. (Barcroft and Edholm, 1945). The intravenous infusions were maintained at a rate of 4 ml./min. by means of a mechanically driven syringe. During a 10-minute control period 0.9% NaCl (with ascorbic acid 0.001%) was infused and blood flow records obtained at half-minute intervals; adrenaline (*l*-adrenaline tartrate, B.D.H.) or noradrenaline ("Levophed," Bayer) made up in this solution was then infused for 10 minutes at a rate of 10 $\mu$g. or 20 $\mu$g./min. The ascorbic acid saline solution was then resumed for 10 minutes.

In the first group of experiments the expired air was collected in Douglas bags, one five-minute sample being taken before, two during, and one after the drug infusion. The volume of each of the samples was measured and the $CO_2$% and $O_2$% determined with a Scholander gas analysis apparatus; the oxygen consumption during each period was calculated and expressed as ml. used per minute. The estimations were accurate to $\pm 10$%.

In the second group of experiments the alveolar air was collected continually by means of a sampler modified from that described by Rhan and Otis (1949). The last 10 ml. of each expiration was drawn through an infra-red $CO_2$ analyser by an air-pump; the sampling was controlled by a valve, operated by an electro-magnetic relay device, which was triggered by an inspiratory valve in the nose-piece. The samples were therefore of end-expired air, but during quiet breathing in these experiments the $CO_2$% values were very close to those obtained by the Haldane-Priestley sampling of alveolar air. Readings on the analyser were taken every 10 seconds and corrected for the slow loss in sensitivity of the instrument throughout the experimental period.

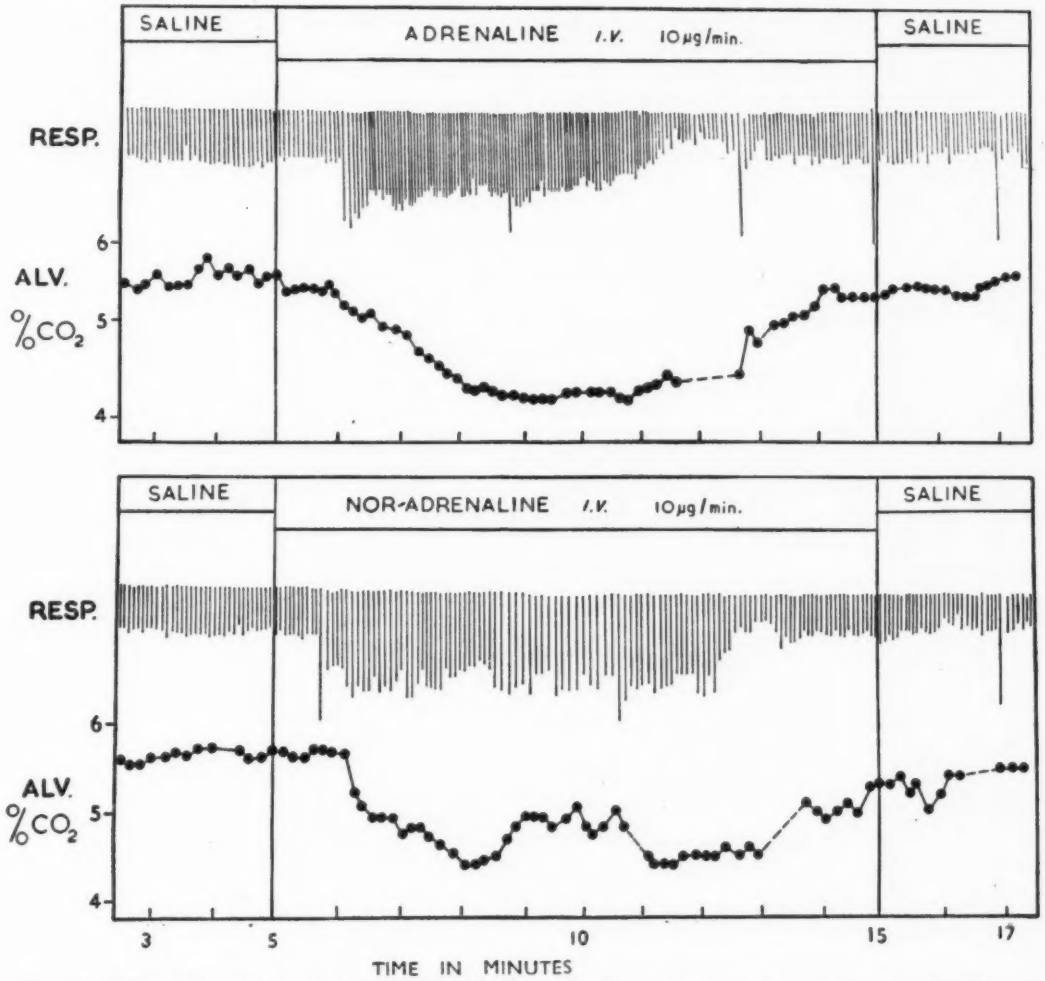


FIG. 1.—Stethograph records and alveolar air $CO_2$ % during intravenous infusions of adrenaline and noradrenaline, at the rate of 10 µg./min. for 10 min., in subject R.W.

## RESULTS

Fig. 1 shows two stethograph records, one taken during adrenaline and the other during noradrenaline infusion. In both there is a marked increase in tidal volume, and there is an increase in respiratory rate with adrenaline. The increase in rate was not present in every experiment, but an increase in depth was always found with both drugs. The increase in tidal volume was often preceded by a few sighing inspirations, and was greatest during the first five minutes of the infusion. The transient shallow respiration which follows the hyperpnoea in these two records was observed in about one-third of the subjects.

Fig. 2 shows the average of six experiments with adrenaline and six with noradrenaline in which the respiration rate, respiratory minute volume, and oxygen consumption are compared together with measurements of the forearm blood flow. The blood flow was recorded, as it gave an objective measure of the subject's response to the drug infused. The response to adrenaline followed the normal pattern (Allen, Barcroft, and Edholm, 1946) with an initial transient vasodilatation followed by a sustained increase in flow ; noradrenaline often produced a small transient increase in flow at the beginning of the infusion, but there was no sustained vasodilatation.

The average increase in respiratory rate was small with both adrenaline and noradrenaline, but the minute volume was always conspicuously increased. This increase in minute volume was greatest during the first five minutes of the infusion, and was larger with adrenaline than with noradrenaline.

These changes in respiratory pattern were accompanied by alterations in the respiratory exchange. Adrenaline increased the oxygen con-

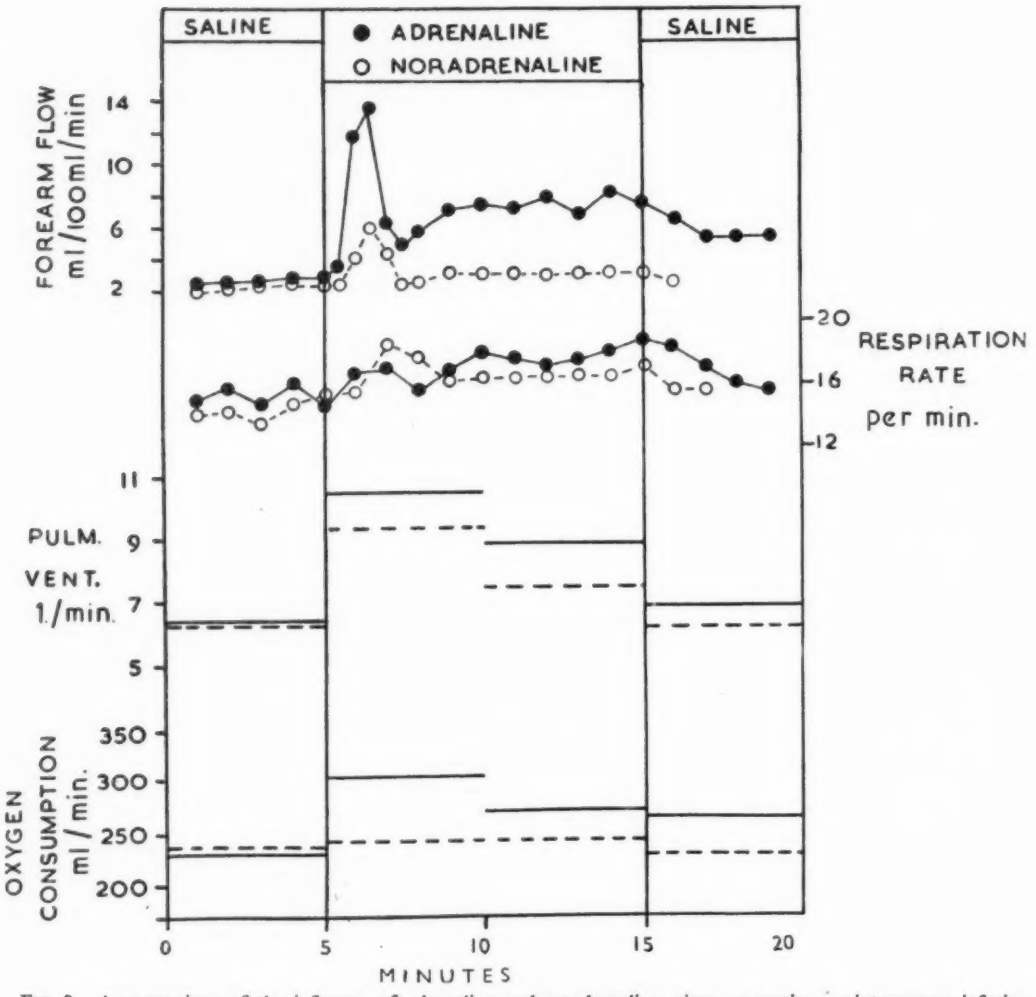


FIG. 2.—A comparison of the influence of adrenaline and noradrenaline, given as continuous intravenous infusion at the rate of 20 μg./min. for 10 min., on forearm blood flow, respiratory rate, pulmonary ventilation, and oxygen consumption in man. The values are the average of six experiments with adrenaline and six with noradrenaline.

sumption by an average of 32% during the first five minutes of the infusion (Table I). The increase was not so marked during the second five-minute period; in four of the subjects there was a fall towards the resting value, while in two the increase was maintained. The effect of noradrenaline on the oxygen consumption was variable; three subjects showed an increase comparable with that found with adrenaline, one a fall, and two no change, during the first five minutes. In one subject only was there a definite increase in oxygen consumption during the second five-minute period.

In the second group of experiments the alveolar air was sampled in 10 subjects during adrenaline infusions. A fall in the $CO_2$% was recorded in seven of these; it remained unaltered in three. A fall in the alveolar $CO_2$% was also observed in five out of six subjects receiving noradrenaline infusions. This fall in alveolar $CO_2$ started during the second minute of the infusions, and the minimum value was reached between the fourth and fifth minute. The alveolar $CO_2$ rose gradually during the second five-minute period of the infusion, and was frequently at the resting level during the last minute of the infusion. Fig. 1 shows the alveolar $CO_2$ figures which were obtained at the same time as the stethograph records for subject R. W. The values cannot be regarded as absolute due to the lag in the galvanometer of the infrared analyser.

## DISCUSSION

The observed stimulation of respiration by adrenaline in man is in keeping with the results of Tompkins, Sturgis, and Wearn (1919), Lyman, Nicholls, and McCann (1923), and Courtice, Douglas, and Priestley (1939), who gave compara-

TABLE I

A COMPARISON OF THE STIMULATING ACTION OF INTRAVENOUS ADRENALINE AND NORADRENALINE ON THE PULMONARY VENTILATION AND OXYGEN CONSUMPTION DURING THE FIRST (A) AND SECOND (B) FIVE-MINUTE PERIODS OF INFUSION. THE VALUES ARE EXPRESSED AS % CHANGE FROM THE RESTING VALUES

| Drug | Subject | % Increase Pul. Vent. | | % Change $O_2$ Cons. | |
|---|---|---|---|---|---|
| | | A | B | A | B |
| Adrenaline 20 μg./min. | J.A. | 153 | 87 | +51 | +33 |
| | M.Y. | 44 | 8 | +24 | −6 |
| | R.L. | 51 | 31 | +28 | +27 |
| | R.B. | 54 | 44 | +15 | +35 |
| | P.B. | 121 | 87 | +56 | +3 |
| | R.P. | 13 | 7 | +19 | +13 |
| | Average: | 73 | 44 | +32 | +18 |
| Noradrenaline 20 μg./min. | S.B. | 59 | 22 | +21 | +9 |
| | R.W. | 40 | 13 | +31 | — |
| | R.B. | 39 | 13 | +2 | +13 |
| | J.A. | 42 | 18 | +16 | −12 |
| | P.B. | 70 | 19 | 0 | +7 |
| | R.P. | 44 | 40 | −23 | +3 |
| | Average: | 49 | 21 | +8 | +4 |

tively small doses by injection. Cori and Buchwald (1930) used continuous intravenous infusions, and found a slight increase of the respiration rate with doses one-tenth of the value of those used in the present investigation ; no record of the minute volume was given. Courtice, Douglas, and Priestley attributed their response to lactic acid production, and this may account for some of the respiratory effects with adrenaline seen by other workers. However, the rapid and abrupt onset of the respiratory responses to both adrenaline and noradrenaline described in this paper must be independent of lactic acid production : Bearn, Billing, and Sherlock (1951) showed that the blood lactate was not significantly raised until 10 minutes after the beginning of a continuous infusion of adrenaline of the same size as in the present experiments, and with noradrenaline there was no lactic acid production. The "adrenaline apnoea" seen in the anaesthetized animal is due to the large pressor response produced by the big doses of adrenaline used ; the stimulating action of adrenaline in the anaesthetized animal was observed by Wright (1930) when the blood pressure rise was prevented by a compensator. Small doses of adrenaline were found by Boothby and Sandiford (1923) to stimulate the respiration in unanaesthetized dogs ; Nice, Rock, and Courtright (1914) and Nice and Neill (1924) obtained similar results in anaesthetized dogs, cats, and rabbits.

The time relationships of the respiratory responses to the beginning of the infusion were the same for adrenaline and noradrenaline. The pattern was characterized firstly by the abrupt onset of the hyperpnoea which was maintained, though not at the original level, throughout the first five minutes ; and secondly by the diminished extent of the responses during the second five minutes of the infusion. The onset of the increased rate and depth of respiration, accompanied by the fall in alveolar $CO_2$%, occurred 1 to 1½ minutes after the beginning of the infusion, corresponding in time with the initial changes in forearm blood flow. The initial changes in heart rate and in arterial blood pressure caused by adrenaline and noradrenaline infusions begin at the same time (Allen *et al.*, 1946 ; Barcroft and Konzett, 1949). It is of some interest that all of these responses to adrenaline and noradrenaline, both respiratory and cardiovascular, are at their maximum between the second and the fifth minute of infusion, while they tend to diminish during the second five-minute period, though remaining above their resting levels.

The increased oxygen consumption produced by adrenaline in every subject confirms the frequently observed calorigenic effect of this hormone. The inconsistent calorigenic response to equivalent doses of noradrenaline is in keeping with the results of Reale, Kappert, Skoglund, and Sutton (1950) ; Bearn, Billing, and Sherlock (1951) also showed that noradrenaline had only a small effect on splanchnic oxygen consumption. *In vitro* Sutherland (1950) found that noradrenaline was less effective than adrenaline in releasing glucose from glycogen in tissue slices. The fact that the average increase in the minute volume with noradrenaline was 75% of that with the same dose of adrenaline indicates that the increased oxygen consumption in these experiments cannot be attributed solely to increased activity of the respiratory muscles. The difference between the metabolic effects of adrenaline and noradrenaline also suggests that the stimulation of respiration, observed to approximately the same degree in both, is not due to any general increase in metabolism.

The fall in alveolar $CO_2$ also observed by Lyman, Nicholls, and McCann (1923) can be attributed to the increase in minute volume, for both are at their maximum during the first five minutes of the infusion period. As with all other respiratory stimulants, with the exception of $CO_2$ itself, the stimulation of respiration by adrenaline and noradrenaline proceeds in spite of the diminished alveolar $CO_2$ tension which may fall as low as 32 mm. Hg. This low $CO_2$ tension may be responsible for the short period of shallow respiration sometimes seen during the latter part of the infusion.

## Summary

1. In the normal human subject both adrenaline and noradrenaline stimulate the respiration to a comparable degree when given intravenously at the rate of 10–20 $\mu$g./min.

2. There is an increase in tidal volume, an increase in respiratory minute volume, and accompanying this a fall in the alveolar $CO_2$%. The respiratory rate was usually, but not always, increased

3. The respiratory responses to both adrenaline and noradrenaline are characterized firstly by the abrupt onset of the hyperpnoea, and secondly by the gradual diminution of the response throughout the infusion period.

4. The adrenaline infusions increased the oxygen consumption by an average of 32%, while noradrenaline caused no consistent change. It is suggested, therefore, that the hyperventilation produced by adrenaline and noradrenaline is independent of any general increase in metabolic rate.


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# QUANTITATIVE MEASUREMENT OF THE HISTAMINE-RELEASING ACTIVITY OF A SERIES OF MONO-ALKYL-AMINES USING MINCED GUINEA-PIG LUNG

BY

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A wide variety of compounds have been reported to release histamine from tissues, but there is no common basis for comparing their activity. They have been tested on different tissues and by different methods: in some investigations (MacIntosh and Paton, 1949 ; Collier and Macauley, 1952) an effect of the released histamine, such as skin wealing, or delayed blood-pressure response, has been used to indicate histamine release, in others the released histamine has been directly measured by pharmacological or chemical methods (Alam, Anrep, Barsoum, Talaat, and Wieninger, 1939 ; Rocha e Silva and Schild, 1949 ; Feldberg and Paton, 1951 ; McIntire, Roth, and Sproull, 1951). For the purpose of a quantitative comparison of histamine-releasing activity it is desirable that not only the amount of histamine released but also the concentration of the releaser at the site of action should be known. In previous experiments in this laboratory (Mongar and Schild, 1952) a method was used in which pieces of isolated tissue were immersed in a known concentration of releaser, and the amount of histamine diffusing out in a given time was measured. A disadvantage of this method is that, owing to the thickness of the tissues, the releaser fails to attain diffusion equilibrium inside. Thus, although the concentration of the releaser at the outside is known, its concentration in the interior is undetermined. In the present investigation this disadvantage has been largely overcome by the use of minced tissues.

Tissues are minced into small particles, suspended in Tyrode solution, and stirred. Despite the stresses involved in mincing, the particles maintain their histamine content and show a very low and constant rate of spontaneous histamine loss. When an active substance is added to this material it releases histamine in a reproducible way and at a rate that can be readily measured. We have investigated the effect of concentration and of time on histamine release under these conditions and have used as a measure of activity the concentration of releaser producing a given percentage depletion of histamine in a given time.

As an example of the use of this method the activity of a homologous series of straight-chained mono-amines, $C_nH_{2n+1}NH_2.HCl$, has been compared with that of a well-established releaser. The $C_8$ to $C_{12}$ compounds of this series were found to be considerably more active in releasing histamine from guinea-pig lungs than compound 48/80 (Baltzly, Buck, de Beer and Webb, 1949) which had been previously shown by Feldberg and Paton (1951) to be the most active releaser known.

## METHODS

Guinea-pig lung tissue was disintegrated by a small Latapie mincer (volume about 7 ml.) shown in Fig. 1. The tissue was forced through small holes in a perforated steel disc by advancing the plunger. At the same time rotating blades cut off the extruded material into small pieces. A comparatively coarsely minced tissue was obtained by using a disc with 1.5 mm. diameter holes and blades rotating so as to cut off pieces of 0.9 mm. length. A more finely minced tissue could be obtained from the coarse mince by using a disc with holes of 0.5 mm. diameter and cutting off at an average length of 0.4 mm. In practice, the pieces were not uniform and contained strands of connective issue. The particles of the coarse mince had an average weight of about 2 mg. and the fine material of about 0.25 mg. Most of the connective tissue (10% by weight of the original material) did not pass through the perforated disc. The minced tissue was washed on a coarse sintered glass filter for 10 to 15 minutes. This treatment resulted in a loss of weight of about 25%. The washing solution remaining in the minced tissue was removed by gentle suction, leaving a material which could be divided into a number of uniform samples for histamine release.

The material was divided into 12 uniform samples by means of the device shown in Fig. 1, consisting of a perspex disc 1.2 cm. thick with 12 holes of 7 mm.

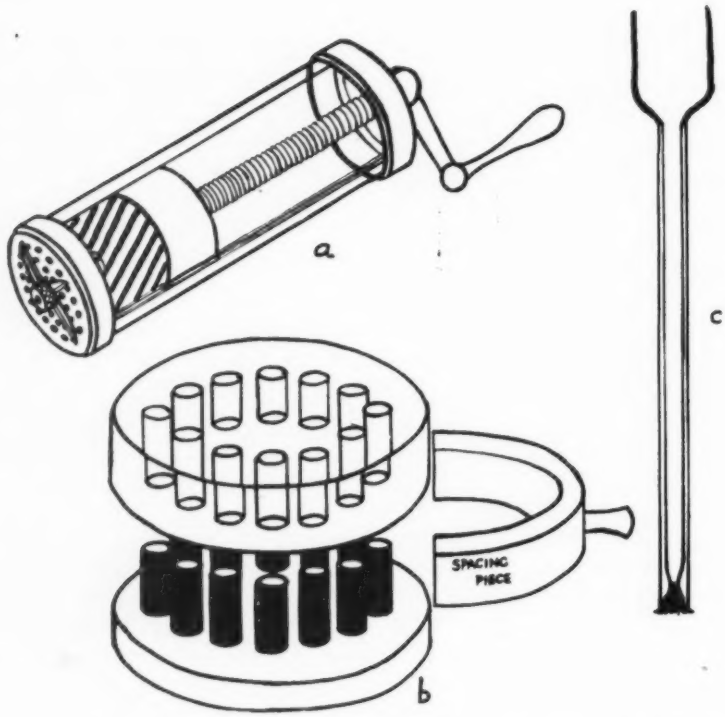


FIG. 1.—Apparatus used in connection with histamine release from minced tissues: (*a*) Mincer producing tissue particles of 2 mg. average weight. (*b*) Perspex sampler for producing twelve uniform samples of equal volume, 0.05 to 0.2 ml., according to the thickness of spacing pieces. (*c*) Filter tip of pipette for withdrawing liquid from a suspension of tissue particles. The filter pad consists of teased cotton-wool.

diameter. A second disc with 12 plungers fitting into the holes was used to close the bottom of the holes at a known depth as determined by the spacing pieces. The holes were then filled with the minced tissue level with the surface of the top disc. In this way 12 samples of identical volume and composition were obtained which could be extruded by removing the spacing pieces and pressing the two discs together.

The cylinders of minced tissue were transferred with a spatula to 10 ml. beakers containing 2 ml. Tyrode solution. The beakers were immersed in a bath at 37° C. and rocked from side to side through an angle of 60 degrees every second. An 8-mm. glass ball rolling across the bottom of each beaker gave efficient stirring without splashing particles of tissue on to the sides of the beaker above the level of the liquid. After 15 to 30 minutes at 37° C. the Tyrode washing solutions were removed and immediately replaced by 1-ml. portions of warm Tyrode solution containing different concentrations of histamine releasers, except for one beaker which was used as a control to determine the spontaneous rate of release of histamine.

To remove solutions from the minced tissue in the beaker a 5-ml. pipette with filter tip was used, consisting of a small cup plugged loosely with about 1 mg. cotton-wool as shown in Fig. 1. The end of the pipette was placed firmly on the bottom of the beaker, care being taken not to trap particles of tissue under it. Gentle suction then drew off most of the liquid without allowing the filter to get clogged. The remaining liquid adhering to the minced tissue was removed by gently stirring the contents of the beaker with the end of the pipette, keeping the tip pressed on the bottom, and sucking to remove the liquid so expressed. Finally the top of the pipette was closed and the filter pad along with any particles of tissue adhering to it returned to the beaker. This method of stirring and removing the fluid was evolved after a certain amount of experimenting. For example, an attempt was made to have a sintered glass filter permanently fitted in the bottom of a funnel-shaped beaker, and remove the test solution through it. This was not found practicable, since the particles tended to clog the filter. Neither was it possible to effect stirring by bubbling oxygen through the sintered glass filter because of excessive frothing.

The solutions containing the released histamine were boiled to stop bacterial action, and assayed on the guinea-pig ileum using a four-point method and automatic apparatus (Mongar and Schild, 1950). The histamine remaining in the minced tissue at the end of the experiment was released by boiling for three minutes in Tyrode solution, and similarly assayed. Control tests showed that grinding with sand was not necessary. Although the mono-amines produce some depression of the histamine response of the guinea-pig ileum, this was not a marked feature of the present experiments owing to the small volume of release solution used (1 ml. solution to 0.2 ml. tissue) which could be diluted as much as 100 times before assay. When depression occurred it was controlled by the addition of an equal concentration of mono-amine to the histamine standard. Control tests with atropine and mepyramine indicated that the activity of the test solutions was due to histamine.

Methylamine and ethylamine (B.D.H.) were used as the hydrochlorides. The higher amines, *n*-propylamine to *n*-decylamine, were supplied by Light & Co., and *n*-dodecylamine to *n*-octodecylamine by Armour & Co. (redistilled quality) as bases from which the hydrochlorides were prepared. 48/80 was obtained through the kindness of Dr. Kellaway, of the Wellcome Foundation.

## RESULTS

### *Histamine in Minced Tissues*

The histamine content of minced lung particles was found to be similar to that of whole lung. There was a loss of histamine on washing the coarse minced tissue with Tyrode solution, but there was a corresponding loss of weight of the tissue due to disintegration products passing through the filter so that the histamine content per unit weight of tissue remained the same. Similarly, there was no marked reduction of histamine content due to putting the coarse particles through the fine mincer. The results of a typical experiment are given in Table I, which shows the histamine content of (*a*) coarse lung particles immediately after mincing, (*b*) the same material after washing for 15 minutes with a large volume of Tyrode solution, and (*c*) the material after it has been put through the fine mincer and again washed. Although the weight of tissue is reduced to one-half, the histamine per gramme of tissue is reduced by only 16%. Histamine loss is apparently only from disrupted cells, the contents of which are mostly removed along with the histamine.

TABLE I

EFFECT OF MINCING ON HISTAMINE CONTENT OF GUINEA-PIG LUNG TISSUE

| | Wt. of Tissue (g.) | Histamine Content ($\mu$g./g.) |
|---|---|---|
| (*a*) Coarse lung particles immediately after mincing | 3·5 | 19 |
| (*b*) The same material after washing for 15 minutes .. | 2·5 | 19 |
| (*c*) The material after fine mincing and washing .. | 1·8 | 16 |

It was of interest to find out whether further mechanical disintegration of the cells would release all the histamine in the tissue as had previously been found for dogs' liver by Trethewie (1938). The finely minced tissue was treated in a Potter blender and the concentration of free histamine in the suspension was measured at various times during treatment. Fig. 2 shows that all the histamine could be released by this mechanical method, since no further release was obtained when the suspension was heated in a boiling-water bath for three minutes.

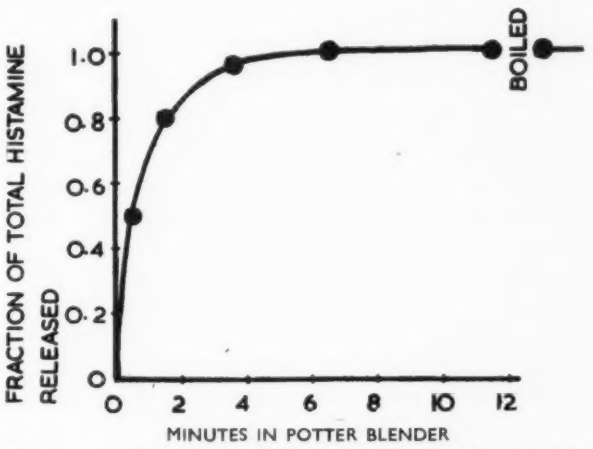


FIG. 2.—Complete release of histamine from minced lung tissue by mechanical disintegration (Potter blender and sand). Samples of the suspension were removed at intervals and immediately assayed.

### *Histamine Release from Minced Tissues*

*Spontaneous Release.*—The rate of loss of histamine from minced unwashed tissue when suspended in successive lots of Tyrode solution was measured. The spontaneous rate of loss is high for the first 20 minutes, amounting to about 20% of the total histamine content. After 30 minutes the spontaneous rate of loss is small and almost constant at about 0.1%/min. (Fig. 3).

*Action of Chemical Releasers.*—Fig. 4 shows the shape of a typical release curve from minced tissue using compound 48/80 at a concentration of $10^{-3}$.

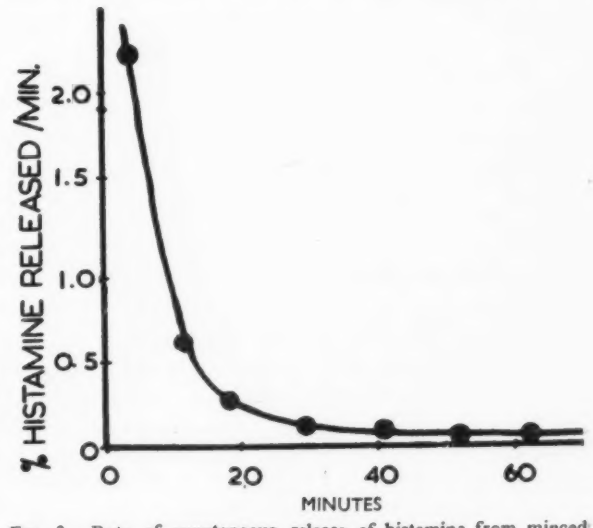


FIG. 3.—Rate of spontaneous release of histamine from minced guinea-pig lung tissue.

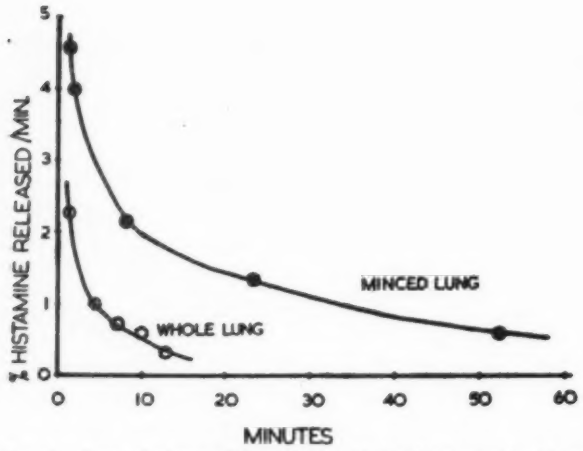


FIG. 4.—Rate of release of histamine by $10^{-3}$ 48/80 from minced and whole guinea-pig lung tissue.

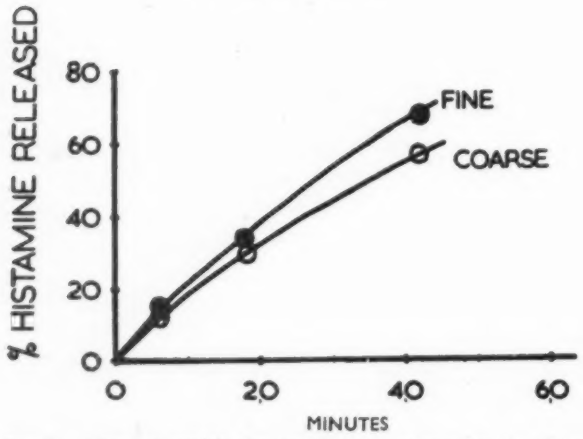


FIG. 5.—Effect of particle size on histamine release by $10^{-3}$ 48/80 (upper curve, average particle weight: 0·25 mg. Lower curve, average particle weight: 2 mg.)

Although the initial rate of release after adding 48/80 is over 50 times the spontaneous rate, the liberation of histamine is by no means instantaneous; it takes up to an hour for the release to be substantially completed. The rate of release from pieces of whole lung weighing about 100 mg. is shown for comparison (Mongar and Schild, 1952). It will be seen that release from minced tissue is much greater than for an equal weight of whole lung and continues to be measurable for a longer time.

*Comparison of Coarse and Fine Mince.*—Fig. 5 shows the time course of release from coarsely and finely minced tissue. Although the average particle weight of fine mince is only about one-eighth that of coarse mince, the rate of histamine release is not much greater. These results indicate that diffusion through tissue spaces does not determine the rate of release in minced tissues, hence little is gained by reducing particle size further by fine mincing. When whole tissues are used less histamine is released due to incomplete diffusion of the releaser, whereby an effective concentration is only attained in the superficial layers of the tissue.

*Effect of Stirring.*—Fig. 6 shows the effect of stirring by (1) rocking the beaker from side to side, and (2) increasing agitation by means of a glass

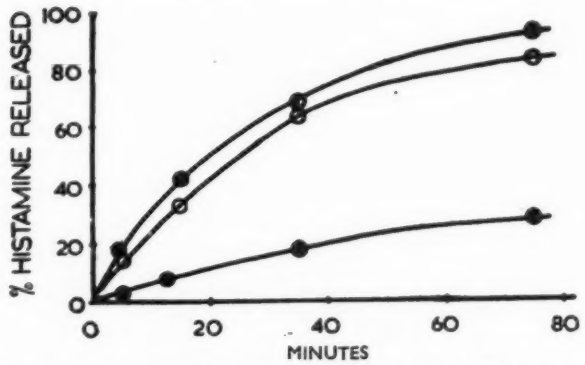


FIG. 6.—Effect of stirring on histamine release by $10^{-3}$ 48/80. ● = No stirring (bottom curve). ○ = Rocking beaker (middle curve). ◉ = Rocking beaker and g'ass ball (top curve).

ball rolling about on the bottom of the rocking beaker. These effects are compared with the rate of release obtained when the particles of tissue were suspended in Tyrode without stirring; thorough stirring is necessary to obtain maximal and reproducible rates of release.

*Reproducibility.*—Using the methods of subdivision, sampling, and stirring described, six replicate experiments gave satisfactory reproducibility as shown in Table II. A method is thus provided by which twelve or more experiments

TABLE II

UNIFORMITY OF HISTAMINE CONTENT AND REPRODUCIBILITY OF HISTAMINE RELEASE IN 6 PARALLEL SAMPLES OF MINCED GUINEA-PIG LUNG

| Sample: | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Total histamine in 0·2 ml. sample (μg.) .. | 2·9 | 2·6 | 2·4 | 2·5 | 2·8 | 2·7 |
| % spontaneous release in 10 min. .. .. | 1·0 | 0·9 | 1·1 | 1·0 | 0·9 | 0·9 |
| % release by $10^{-3}$ 48/80 in 10 min. .. .. | 35 | 33 | 39 | 29 | 32 | 30 |

can be performed on material which is uniform both with regard to histamine content and histamine release.

*Effect of Time and Concentration.*—The rate of release of histamine by different concentrations of (*a*) 48/80 and (*b*) octylamine is shown in Fig. 7. All the histamine in the tissue can be released within 1 hour by $10^{-3}$ octylamine or $10^{-2}$ 48/80.

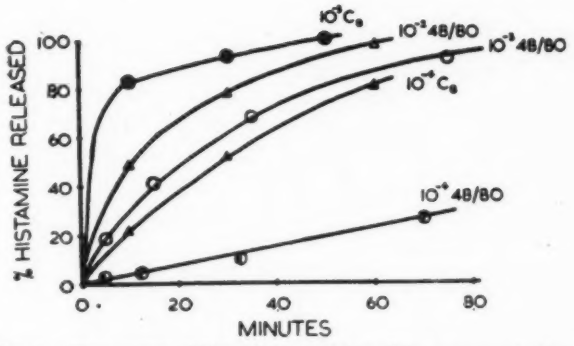


FIG. 7.—Time course of histamine released by different concentrations of 48/80 and of octylamine. Points on each curve were obtained from one sample of tissue by successive replacements of the releaser solution.

With lower concentrations the release is not complete in this time, but the shape of the curves suggests that they tend asymptotically to 100%. The curves are similar to those obtained with *d*-tubocurarine on the intact rat diaphragm (Rocha e Silva and Schild, 1949) except that in the latter preparation the maximal release was only 70% of the histamine in the diaphragm.

*Comparison of Activity.*—The concentration-release curves for a selected time can be used for comparing the activities of these compounds. Fig. 8 shows the curves for 10- and 60-minute release

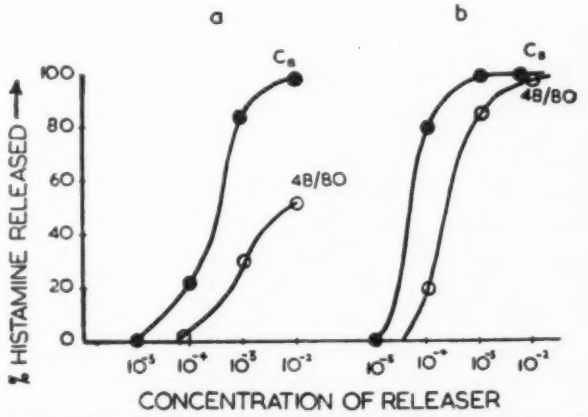


FIG. 8.—Concentration-release curves for octylamine and 48/80 with release times of (*a*) 10 and (*b*) 60 minutes.

times. It will be apparent that as the curves for 10 minutes are not parallel throughout their whole course the relative activities will depend to some extent on the release level chosen for the comparison. By using the longer time the shapes of the concentration-release curves become more nearly alike, and at the level of 25 or 50% release the slopes are sufficiently steep to make possible accurate comparisons. On the other hand, when very long periods for release are used the correction for spontaneous release becomes large: for weak releasers comparisons at the 10% level may have to suffice.

*Activity of Mono-alkylamines*

In view of the high activity of octylamine as a histamine releaser the series of straight-chained mono-amines, $C_nH_{2n+1}NH_2.HCl$, up to $C_{18}$ was tested. Fig. 9*a* shows the concentration-release curves for release in 10 minutes and Fig. 9*b* curves for release in 60 minutes. With a release period of only 10 minutes, the weaker releasers cannot be given in sufficient concentration to produce a substantial effect, but with a period of 60 minutes they can be compared at the 50% release level. The concentrations giving this release are shown in

TABLE III

ACTIVITY OF AMINES $C_nH_{2n+1}NH_2.HCl$ AND 48/80 AS HISTAMINE RELEASERS FROM MINCED GUINEA-PIG LUNG. COLUMN (*a*) GIVES THE CONCENTRATIONS REQUIRED TO PRODUCE A 50% RELEASE IN 60 MIN.; COLUMN (*b*) THE ACTIVITIES RELATIVE TO THE $C_{10}$ MEMBER OF THE SERIES

| Compound | (*a*) | (*b*) |
|---|---|---|
| $C_2$ | $1{\cdot}0\times10^{-3}$ | 7 |
| $C_4$ | $6{\cdot}3\times10^{-4}$ | 11 |
| $C_6$ | $4{\cdot}1\times10^{-4}$ | 18 |
| $C_8$ | $9{\cdot}8\times10^{-5}$ | 74 |
| $C_{10}$ | $7{\cdot}2\times10^{-5}$ | 100 |
| 48/80 | $4{\cdot}7\times10^{-4}$ | 15 |

Table III. $C_{10}$ is 14 times more active than $C_2$ and nearly seven times more active than 48/80.

Fig. 10 shows the amount of histamine released in ten minutes by equimolar concentrations of mono-amines of chain length $C_1$ to $C_{14}$; $C_{10}$ is the most active compound. Reducing the chain length to six carbon atoms or increasing it to 14 greatly reduces or abolishes the releasing activity. Compounds of chain length greater than $C_{14}$ cannot be tested at a concentration of $5\times10^{-4}$ M owing to their low solubility; but a saturated solution of $C_{18}$ (about $10^{-4}$ M) was inactive. This contrasts with the results of McIntire *et al.* (1951), who found that $C_{18}$ was highly active in releasing histamine from rabbits' blood, whereas $C_{12}$ was almost inactive.

## DISCUSSION

It has been shown that minced guinea-pig lung provides suitable material for testing the activity of histamine releasers. A number of uniform samples may be prepared from this material for testing the activity of different drugs or different concentrations of the same drug. All the histamine present in the tissue can be released using this method, in contrast to previous diffusion methods using intact tissues in which only a fraction of the

histamine was liberated. The essential feature of this type of experiment is that the particles are large enough for most of the cells to remain intact and so retain their histamine, yet small enough to allow rapid diffusion of the releaser to all the cells. Since the rate of histamine release from minced tissue varies according to the concentration of releaser, and the concentration-action curves are generally steep and parallel, quantitative comparisons of activity are possible.

Tested in this way, the straight-chained mono-amines octylamine to dodecylamine are powerful histamine releasers considerably more active than 48/80. The maximum activity of the mono-amine with 10 carbon atoms is reminiscent of the finding of MacIntosh and Paton (1949) that in a series of diamines maximum histamine-releasing activity occurs with a similar chain length. Since many of the active histamine releasers previously tested, such as *d*-tubocurarine, compound 48/80, the diamidines, and the diguanidines, also have at least two basic groups separated by a large hydrocarbon moiety, it seemed reasonable to assume that their activity was due to the goodness of fit of two basic groups for bifunctional receptors in the tissue. Such an explanation is clearly inapplicable to the mono-amines which have only one basic group attached to the hydrocarbon chain, the length of which alters the hydrophobic nature of the molecule rather than any goodness of fit. Each carbon atom is capable of free rotation, so there will be a whole series of configurations for the chain, unlike that for a molecule with two polar groups of equal sign at the extremities which will tend to maintain it in a fully extended configuration. The maximum activity of decylamine may be the resultant of two opposing processes: increasing activity with increasing chain length opposed by an increasing tendency to form micelles with increasing chain length. The aggregation of molecules in solution would reduce the effective concentration of releaser. This explanation for the effect of chain length of activity is supported qualitatively by conductivity measurements (Ralston and Hoerr, 1942) which show that the $C_{10}$ member of the series is the first one to show micelle formation.

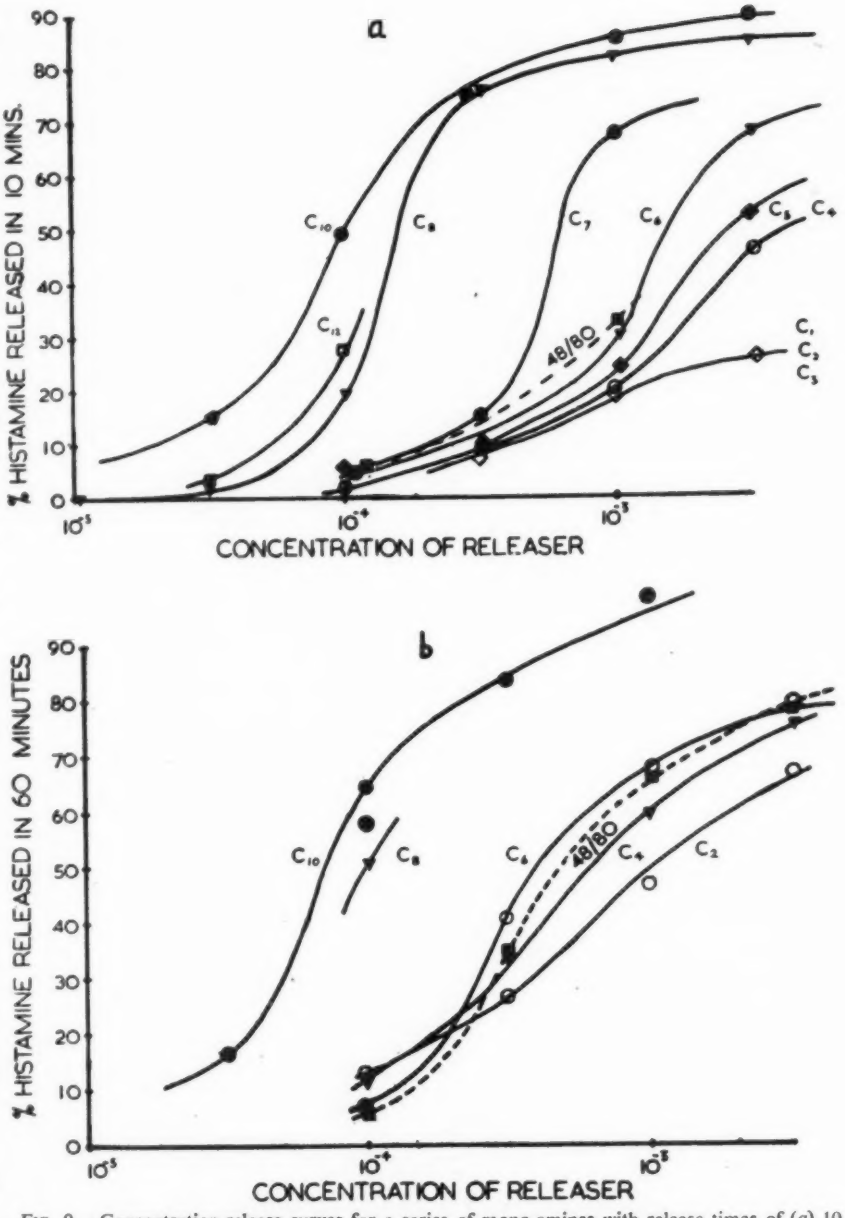


FIG. 9.—Concentration-release curves for a series of mono-amines with release times of (*a*) 10 minutes, (*b*) 60 minutes. The curves in (*a*) were obtained in separate tests from those in (*b*). Points in (*b*) are the results of single experiments, points in (*a*) represent the mean of several experiments.

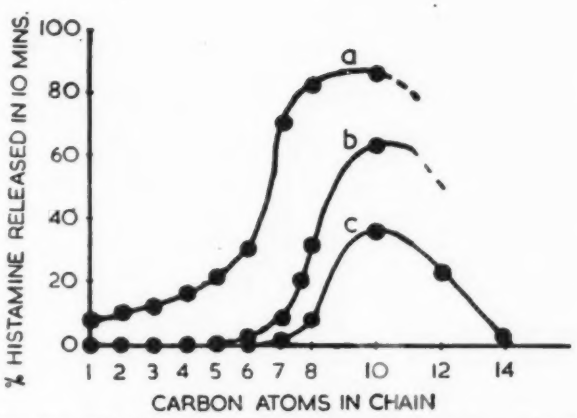


FIG. 10.—Effect of chain length on histamine release from minced guinea-pig lung by equimolar solutions of mono-amines $C_nH_{2n+1}NH_2.HCl$: (*a*) $10^{-2}$M, (*b*) $10^{-3}$M, (*c*) $5\times10^{-4}$M.

In unpublished experiments we have found that the mono-amines produce a triple response in human skin in concentrations similar to those needed for histamine release from minced guinea-pig lung. The effect of chain length on activity was also similar. On the other hand, the concentration of 48/80 required to produce wealing (about $10^{-7}$) was very much less than that required for release from isolated tissues (about $10^{-4}$), so that on the basis of skin tests 48/80 appears much more active as a histamine releaser. It cannot be stated at present whether these discrepancies are due to a fundamental difference between the two methods of assessing histamine-releasing activity (the one direct, the other indirect) or to tissue or species specificity.

## SUMMARY

1. A method is described for obtaining particles of guinea-pig lung of 0.25 or 2 mg. average weight, and of dividing the material into a number of samples of equal histamine content.

2. This material has a histamine content similar to that of whole lung, and when suspended in Tyrode solution it shows a very low spontaneous rate of loss of histamine. When a histamine releaser is added, it releases all its histamine into the surrounding fluid, the rate of release depending on the concentration of releaser.

3. The concentration-release curves for a given time of contact are generally steep and parallel, and are therefore suitable for quantitative comparisons of the activity of different histamine releasers. The concentration of releaser needed to liberate half the histamine in 60 minutes has been chosen as a convenient index of activity.

4. The method has been used to measure the relative activity of the series of mono-amines $C_nH_{2n+1}NH_2.HCl$ up to $C_{18}$, and of compound 48/80. Decylamine was the most active member of the series. It was 14 times as active as ethylamine and nearly seven times as active as compound 48/80.

We wish to thank Miss Betty Harris for technical assistance.

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# OBSERVATIONS ON THE SPECIFICITY OF THE INHIBITION OF CHOLINESTERASES BY TRI-*ORTHO*-CRESYL PHOSPHATE

BY

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(RECEIVED OCTOBER 29, 1952)

In 1941 Bloch demonstrated that horse serum cholinesterase was inhibited by tri-*ortho*-cresyl phosphate (TOCP). He suggested that the characteristic flaccid paralysis observed in TOCP poisoning might be due to inhibition of the cholinesterase at the motor end-plates, leading to a local accumulation of acetylcholine. Hottinger and Bloch (1943) showed that acetylcholine hydrolysis by human and rabbit serum, liver, and brain was also inhibited by TOCP ; tributyrinase activity in serum and liver was also diminished, but alkaline phosphatase, trypsin, arginase, histidase, and pancreatic lipase were not affected. The demyelination observed peripherally and centrally in animals and men poisoned with TOCP, and the delay of about 14 days before the onset of symptoms, are difficult to explain on the basis of Bloch's suggestion that the causative lesion is cholinesterase inhibition at the motor end-plates. When this early work was done, selective substrates for the true and pseudo-cholinesterases were not employed. As Bloch's view implies an inhibition of the true cholinesterase, Earl and Thompson (1952a) studied the effect of TOCP *in vitro* on the true and pseudo-cholinesterases of blood, nervous tissue, and striated muscle from man, rabbit, hen, and rat. In man, the true cholinesterase in erythrocytes, muscle, and nervous tissue was not markedly inhibited by high concentrations of TOCP, but the pseudo-cholinesterases in nervous tissue and in serum were profoundly inhibited by relatively low concentrations. Further, in hens poisoned with TOCP the pseudo-cholinesterases of plasma, brain, and spinal cord were markedly diminished, even one day after poisoning, whereas the true cholinesterase in these tissues was relatively unaffected (Earl and Thompson, 1952b). Mendel and Rudney (1944) had also briefly reported that in the rat oral administration of TOCP lowers the serum cholinesterase but not the true cholinesterase.

From these findings, and since pseudo-cholinesterase appears to be associated particularly with the white fibre tracts in the brain (Ord and Thompson, 1952) and to be the predominant cholinesterase in peripheral myelinated nerves and in the white tracts of the spinal cord (Thompson and Webster, unpublished results), we have suggested (Earl and Thompson, 1952a) that this enzyme may be connected with the maintenance of the myelin sheaths of nerve fibres, and that its inhibition by TOCP might be causally related to the demyelination and consequent paralysis.

We have therefore examined the action of this compound on some enzymes which might be regarded as relevant to this possible action. Since defects in pyruvate metabolism may accompany peripheral neuritis due to thiamine deficiency, or certain other causes, the abilities of brain preparations from normal and TOCP-intoxicated hens to oxidize both glucose and pyruvate have been compared. As adrenaline and noradrenaline are also concerned with transmission processes, and as brain contains an amine oxidase (Pugh and Quastel, 1937 ; Blaschko, Richter and Schlossmann, 1937), the effect of TOCP on the ability of brain homogenates to oxidize tyramine both *in vitro* and *in vivo* has also been studied. Following the demonstration by Jansen, Nutting, Jang, and Balls (1949) that the activity of trypsin and chymotrypsin is inhibited by small concentrations of di*iso*propyl fluorophosphonate (DFP), trypsin has also been examined for TOCP-sensitivity. Finally, experiments have been carried out with lecithinases and with Sloane-Stanley's (1951) cephalin-splitting enzyme from brain tissue.

## METHODS AND MATERIALS

*Pseudo-cholinesterase* activity in homogenates of hen brain and spinal cord has been assayed as

described previously (Earl and Thompson, 1952a), using butyrylcholine perchlorate (0.03 M) as substrate.

*Glucose and Pyruvate Oxidation.*—Extra oxygen uptake of hen brain "brei" was measured by the Warburg technique, using glucose (0.014 M) or sodium pyruvate (0.018 M) as substrate.

*Amine Oxidase* activity was determined by measuring the extra oxygen uptake of hen spinal cord homogenates in the presence of 0.01 M-tyramine hydrochloride (L. Light & Co., Ltd.) as substrate.

*Trypsin* (Hopkin & Williams, Ltd.) activity was measured both by the method of Charney and Tomarelli (1947), and by micro-Kjeldahl estimation of trichloroacetic acid-soluble nitrogen liberated from casein (British Drug Houses, Ltd., light white soluble), using the Markham apparatus (1942).

*Lecithinases.*—(1) A dry preparation of the $\alpha$-toxin of *Cl. Welchii* was used as enzyme source. Activity was measured by a modification of the technique of Zamecnik, Brewster, and Lipmann (1947), using a bicarbonate buffer (*p*H 7.3) containing 0.016 M-$NaHCO_3$ gassed with 95% $N_2$+5% $CO_2$. Approximately 10 LD50 (mouse) of toxin were used in each Warburg flask with a final concentration of 3% ovolecithin as substrate. $CaCl_2$ was not used to activate the system, as it was found that the $CO_2$ output became non-linear in less than 5 min. in the presence of $Ca^{++}$.

(2) A 1-in-6 (w/v) homogenate of rat pancreas in $NaHCO_3$ buffer at *p*H 7.5 with 1% ovolecithin as substrate was used as an alternative enzyme source.

*Brain Cephalinase.*—A 1-in-50 homogenate of guinea-pig brain in Krebs bicarbonate buffer (with $KH_2PO_4$ omitted) was used as enzyme source. Activity was measured under the conditions described by Sloane Stanley (1951), using 4% (w/v) crude cephalin as substrate. All enzyme estimations were carried out in duplicate.

*Tributyrinase* activity was estimated in homogenates of hen spinal cord as described previously (Earl and Thompson, 1952a).

*Butyrylcholine Perchlorate* (BuCh).—Choline perchlorate, prepared from choline chloride (Hopkin & Williams, Ltd.), was refluxed with excess of freshly distilled butyryl chloride (British Drug Houses, Ltd.). Recrystallized from ethanol: m.p. 72–74° (Found: C, 39.5; H, 7.2; N, 5.4; Cl, 12.8. Calc. for $C_9H_{22}O_6NCl$: C, 39.5; H, 7.4; N, 5.1; Cl, 13.0). Micro-analysis by Drs. Weiler and Strauss, Oxford.

*Sodium pyruvate* was prepared from crude pyruvic acid (British Drug Houses, Ltd.) by the method of Robertson (1942).

*Sulphanilamide-azocasein* was prepared by the method of King (1951).

*Lecithin* was prepared from egg yolk according to Macfarlane and Knight (1941).

*Cephalin* was prepared from human brain by the method of Folch (1942).

*Tri*-ortho-*cresyl phosphate* (TOCP) was prepared from pure *o*-cresol (Geigy Pharmaceutical Laboratories, Ltd.).

*Diisopropyl fluorophosphonate* (DFP) was kindly provided by the Experimental Station, Porton.

## RESULTS

*Glucose and Pyruvate Metabolism.*—Experiments were first carried out to determine the effect of TOCP *in vitro* on the oxidation of glucose and pyruvate by brain. The human brain tissue (cerebral cortex) was obtained fresh from operation; whole hen and pigeon brains were used, about 100 mg. of the mixed minced tissue from each species being added to each Warburg flask. No significant effect on oxygen uptake was produced by the addition to the system of TOCP in concentrations up to 167 $\mu$g./ml., whereas a concentration of 67 $\mu$g./ml. produced about 90% inhibition of pseudo-cholinesterase activity in human brain (Earl and Thompson, 1952a).

The results of the *in vivo* experiments are given in Table I. Estimations of the levels of glucose and pyruvate oxidation were carried out in brain preparations from normal hens and from hens killed at intervals up to 19 days after poisoning; in the birds killed 1–8 days after poisoning there were no abnormal signs; in one killed after 12 days there was slight leg weakness, while those killed after 15 and 19 days showed severe weakness of the legs, the wings also being slightly weak. It will be seen that the rate of glucose

TABLE I

OXIDATION OF GLUCOSE AND PYRUVATE BY BRAIN *BREI* FROM HENS POISONED WITH TOCP

(Dose: 1 ml./kg. *per os.*) ($O_2$ uptake measured over 0–30 min.)

| | Days after Poisoning | Extra $O_2$ Uptake ($\mu$l. $O_2$/g./hr.) in presence of | |
|---|---|---|---|
| | | Glucose | Pyruvate |
| Normal hens .. | — | 1,365* | 1,515* |
| | — | 1,140 | 1,115 |
| | — | 790 | 1,175 |
| | — | 940 | 1,095 |
| | — | 1,028 | 1,465 |
| | — | 1,125 | 1,245 |
| | — | 1,315 | 1,655 |
| | Mean: | 1,100 | 1,325 |
| TOCP-poisoned.. | 1 | 1,205 | 1,310 |
| | 2 | 1,415 | 1,390 |
| | 5 | 965 | 1,160 |
| | 8 | 1,295 | 1,325 |
| | 12† | 1,217 | 1,385 |
| | 15‡§ | 985 | 957 |
| | 19§ | 1,495 | 1,705 |
| | Mean: | 1,225 | 1,320 |

* $O_2$ uptake measured over 0–54 min. † Slight weakness of feet and legs. ‡ $O_2$ uptake measured over 0–45 min. § Severe leg weakness; wings slightly affected.

oxidation in the poisoned brains was not significantly affected, 2 of the 7 levels found in the normal birds being lower than any in the poisoned series. Apart from one of the birds killed at 15 days the rate of pyruvate oxidation also agrees well with the rates found for the normal tissue.

In order to ensure that the TOCP had in fact been absorbed and had reached the nervous system, especially in those birds which were killed before the stage of paralysis became manifest, estimation of the pseudo-cholinesterase activity of the brain was also carried out in the poisoned birds. It was found to be significantly diminished in all poisoned birds as compared with previously determined normal values (Earl and Thompson, 1952b).

In support of these findings it should be mentioned that in earlier experiments in collaboration with Dr. C. L. Joiner it was found that the blood pyruvate level was normal in pigeons poisoned with TOCP and showing signs of paralysis. A preliminary report on these observations on pyruvate metabolism in TOCP poisoning has already been made (Thompson, 1952).

*Amine Oxidase.*—Three experiments were carried out on the amine oxidase activity of hen spinal cord, when tyramine hydrochloride was used as substrate. A concentration of TOCP sufficient to produce 95% inhibition of pseudo-cholinesterase activity in this tissue (Earl and Thompson, 1952a) produced no inhibition of amine oxidase activity which could be regarded as significant. In support of these *in vitro* observations we have measured the amine oxidase activity of the spinal cords of three hens poisoned with TOCP; in two of these, poisoned 24 hours before killing by the oral administration of 1 ml. TOCP/kg., the extra $O_2$ consumption found was 184 and 283 $\mu$l./g./hr., and in the third, poisoned 48 hours before killing, 226 $\mu$l./g./hr., i.e., the levels of oxidation were in each hen above the mean found for the three normal birds used for the *in vitro* experiments.

*Trypsin.*—Six experiments were carried out with an impure trypsin preparation (Hopkin & Williams, Ltd.) in order to compare its sensitivities to DFP and TOCP. Varying concentrations of DFP ranging from $10^{-6}$ to $10^{-3}$M were added to tubes containing a final concentration of 0.25% (w/v) trypsin and 1.25% (w/v) sulphanilamide-azocasein in 0.5% $NaHCO_3$ (final *p*H 8.1), and incubated for five minutes at 38°. The rate of proteolysis was determined colorimetrically by addition of 0.5N-NaOH to trichloroacetic acid filtrates of the reaction mixture (Charney and Tomarelli, 1947). The $I_{50}$ value of DFP for trypsin under these conditions was found to be $2.5 \times 10^{-5}$M. Amounts of TOCP up to 2,000 $\mu$g./ml. under the same conditions were found to cause no inhibition.

Two further experiments were carried out using the micro-Kjeldahl technique for the estimation of trichloroacetic acid-soluble N, in which DFP and TOCP were added to a reaction mixture containing 0.025% trypsin and 1.25% casein in 0.5% $NaHCO_3$ (final *p*H 8.4), which was then incubated for 10 minutes at 38°. $10^{-4}$M-DFP produced 86% and 91% inhibition of activity in the two experiments, TOCP again giving no inhibition.

In agreement with Kies and Schwimmer (1942) we have been unable to demonstrate detectable tryptic activity in our brain preparations; our experiments were carried out at *p*H 8.1, and we have not studied the proteolytic system with an optimal activity at *p*H 7.4 described by Ansell, Williams, and Richter (1952).

*Lecithinase.*—In two experiments carried out with a type C lecithinase present in *Cl. Welchii* $\alpha$-toxin under the conditions described above it was found that the addition of 1,000 $\mu$g. TOCP/3 ml. produced no effect. The lecithinase present in rat pancreas was also unaffected by TOCP.

Rat brain was also examined for lecithinase, but we were unable to demonstrate any activity under our conditions.

*Cephalinase.*—We have confirmed the finding of Sloane-Stanley (1951) that guinea-pig brain homogenates contain a cephalin-splitting enzyme; in two experiments we obtained activities, over the first five minutes of the reaction, corresponding to 1,100 and 1,200 $\mu$l. $CO_2$/g. brain/hr., which are of the same order as that reported by Sloane-Stanley. These high rates of reaction declined rapidly, and we found almost linear activities of 42 and 83 $\mu$l. $CO_2$/g./hr. when measured over 20–80 minutes from the start of the reaction.

TOCP in a concentration of 1,000 $\mu$g./3 ml. caused no inhibition of either the initial or the later rates of reaction.

*Tributyrinase.*—In view of Hottinger and Bloch's (1943) report that tributyrin hydrolysis by liver and serum is sensitive to TOCP, Earl and Thompson (1952a) compared the inhibition *in vitro* by TOCP of the tributyrinase of the central nervous system of man and the rabbit with the inhibition of pseudo-cholinesterase. They found that with human tissues concentrations of TOCP which produce 75–99% inhibition of pseudo-cholinesterase in the

nervous system produce only 20–30% inhibition of tributyrinase in these tissues; using rabbit brain, tributyrinase activity was also less sensitive to inhibition than was the pseudo-cholinesterase.

To support these *in vitro* observations, however, we thought it desirable to study tributyrinase levels in poisoned animals. After poisoning hens by a single dose of TOCP (1 ml./kg. by mouth), the birds were killed, the spinal cords removed, and the levels of pseudo-cholinesterase and tributyrinase determined. These levels were then compared with those found in normal birds. The results are shown in Table II. It will be seen that, although the cord tributyrinase is affected, there was never more than 57% inhibition from a "normal" level obtained on the cords from six normal birds. Further, in the same animals the cord pseudo-cholinesterase was always inhibited to a greater degree, the mean of the six experiments giving 45% inhibition of tributyrinase as compared with 70% inhibition of pseudo-cholinesterase.

TABLE II

COMPARISON OF LEVELS OF ACTIVITY OF PSEUDO-CHOLINESTERASE (ChE) AND TRIBUTYRINASE (TB) IN SPINAL CORDS OF HENS POISONED WITH TOCP

(1 ml./kg. *per os*)

| | Days after Poisoning | Activity ($\mu$l. $CO_2$/g./hr.) | | % Reduction from Mean Normal Values | |
|---|---|---|---|---|---|
| | | ChE | TB | ChE | TB |
| Normal (mean) | — | 1,700* | 1,894† | — | — |
| Poisoned | 1 | 786 | 1,232 | 53 | 35 |
| | 1 | 525 | 1,120 | 67 | 41 |
| | 2 | 295 | 812 | 79 | 57 |
| | 2 | 348 | 1,014 | 76 | 46 |
| | 3 | 407 | 1,150 | 73 | 39 |
| | 9 | 471 | 910 | 69 | 52 |
| | | | Mean: | 70 | 45 |

* Mean value obtained from 18 normal birds.
† Mean value obtained from 6 normal birds.

It should be added that we have obtained evidence indicating that the tributyrinase of hen nerve tissue is a distinct enzyme from the pseudo-cholinesterase. Thus, $0.9 \times 10^{-6}$M-eserine produces 82% inhibition of butyrylcholine hydrolysis but no inhibition of tributyrin hydrolysis; further, butyrylcholine and tributyrin together are hydrolysed by brain homogenates at a rate which is 87% of the sum of the rates of hydrolysis of the two substrates separately.

## DISCUSSION

Although only a few enzymes have been studied, this brief survey has demonstrated that, unlike the demyelinating lesions that can accompany vitamin $B_1$ deficiency (Swank, 1940; Street, Zimmerman, Cowgill, Hoff, and Fox, 1941; Swank and Prados, 1942), the development of the demyelination produced by tri-*ortho*-cresyl phosphate is not accompanied by any demonstrable defect in the ability of the nerve tissue to oxidize pyruvate or glucose. It would seem therefore that a fundamentally different biochemical disturbance must initiate and accompany the development of the histological changes.

TOCP was without effect on lecithinase and cephalinase activity, enzymes which might be presumed to play some role in connection with the metabolism of myelin lipids.

Apart from pseudo-cholinesterase, tributyrinase is the only other enzyme which has so far been found to be inhibited by TOCP, but, although homogenates of the spinal cords of hens poisoned with this compound show a partial inactivation of this enzyme, pseudo-cholinesterase activity of the cord appears to be regularly inhibited to a greater degree. Moreover, in human tissues (and man appears to be highly sensitive to poisoning by TOCP) brain tributyrinase is very much less sensitive than pseudo-cholinesterase to inhibition *in vitro* by this substance. Although pseudo-cholinesterase inhibition by TOCP cannot yet be regarded as playing a causative role in the demyelination and paralysis brought about by the ingestion of this compound, it may be concluded that, of the various enzyme systems so far studied in nerve tissue, pseudo-cholinesterase is the one most sensitive to inactivation by tri-*ortho*-cresyl phosphate.

## SUMMARY

1. The effect of varying concentrations of tri-*ortho*-cresyl phosphate on a number of widely different enzyme systems has been studied under both *in vitro* and *in vivo* conditions.

2. Glucose and pyruvate oxidation by brain preparations has been shown to be unaffected.

3. Trypsin, brain amine oxidase, pancreatic lecithinase, and brain cephalinase have also been found to be insensitive to this compound.

4. The tributyrinase activity of the spinal cords of hens poisoned with TOCP has been found to be moderately reduced, but in every experiment the pseudo-cholinesterase of the spinal cord has been inhibited to a greater degree.

We wish to thank Professor G. Payling Wright for kindly providing us with a preparation of *Cl. Welchii* $\alpha$-toxin; Mr. M. F. S. El Hawary for the preparation of pure sodium pyruvate; the Ministry of Supply for

a grant for the purchase of birds; Messrs. Geigy Pharmaceuticals, Ltd., for the gift of the tri-*ortho*-cresyl phosphate; and Messrs. D. H. Burchett and D. E. Jarvis for their skilled technical assistance. Our thanks are also due to the Medical Research Council for a grant to one of us (G. R. W.).

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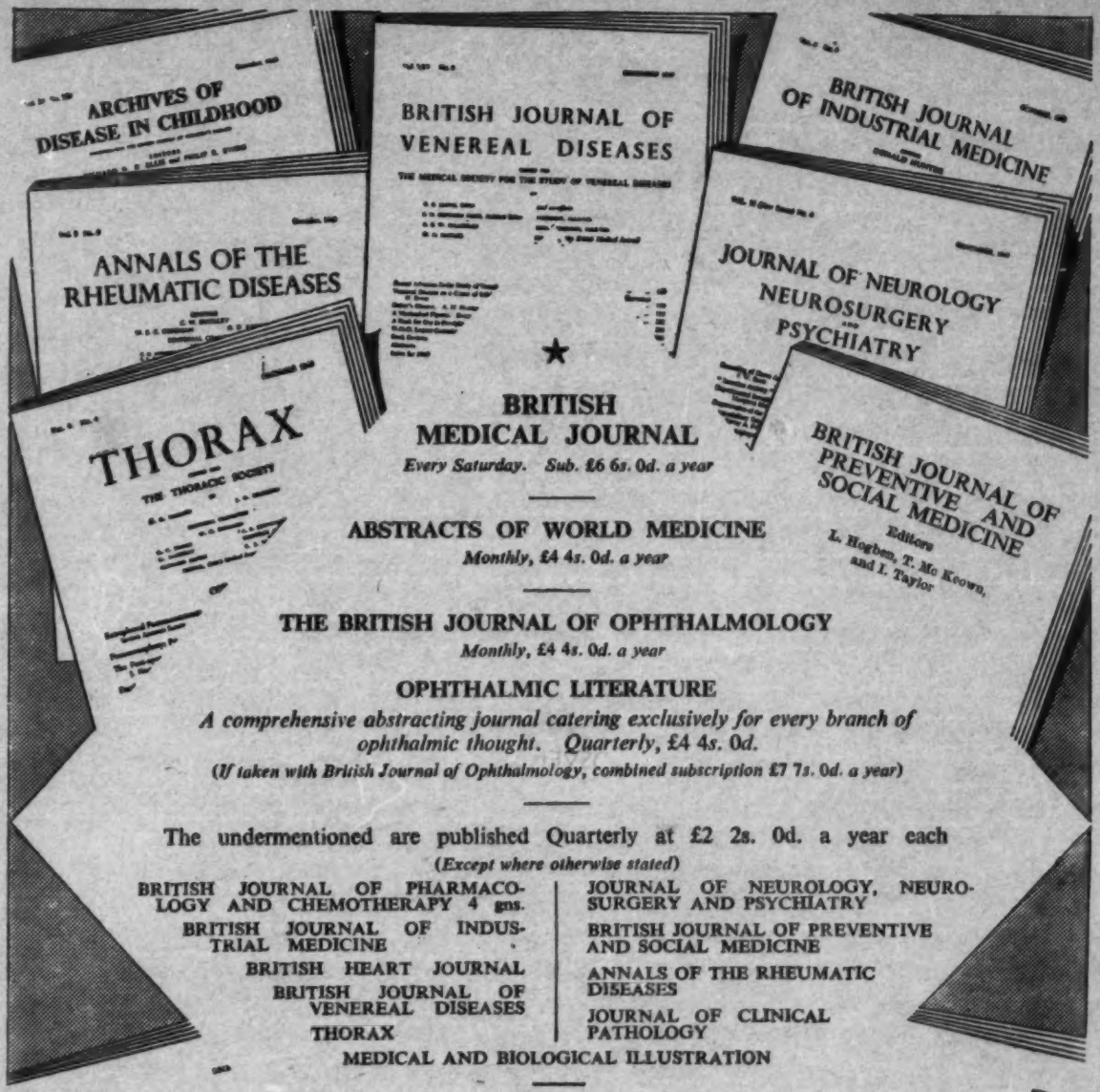
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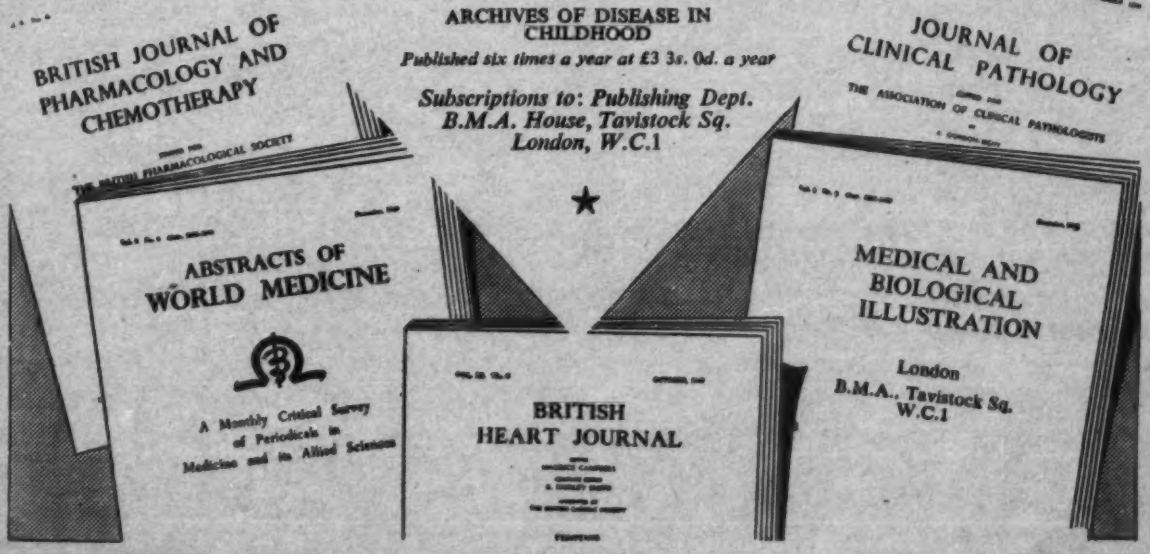
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